



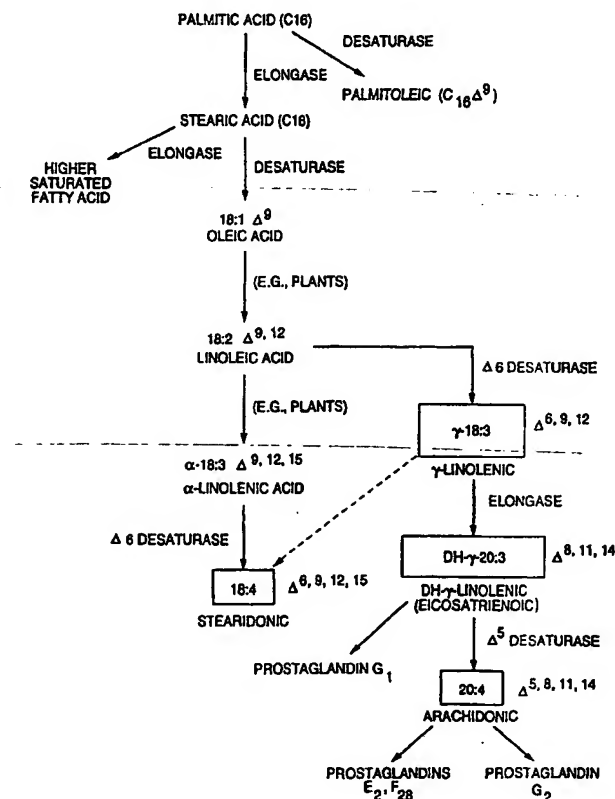
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: ALTERED FATTY ACID BIOSYNTHESIS IN INSECT CELLS USING DELTA FIVE DESATURASE

## (57) Abstract

The present invention relates to a fatty acid  $\Delta 5$ -desaturase able to catalyze the conversion of dihomo- $\gamma$ -linolenic acid to arachidonic acid. Nucleic acid sequences encoding a  $\Delta 5$ -desaturase, nucleic acid sequences which hybridize thereto, DNA constructs comprising a  $\Delta 5$ -desaturase gene, recombinant host insect or animal expressing increased levels of a  $\Delta 5$ -desaturase, purified  $\Delta 5$ -desaturase proteins and antibodies (polyclonal and monoclonal) to the purified  $\Delta 5$ -desaturase proteins are described. Methods for desaturating a fatty acid at the  $\Delta 5$  position and for producing arachidonic acid by expressing increased levels of a  $\Delta 5$  desaturase are disclosed. Methods of producing transgenic oil in microorganisms are disclosed. Methods of purifying the oils and preparing the oils for food products are also disclosed. Fatty acids, and oils containing them, which have been desaturated by a  $\Delta 5$ -desaturase produced by recombinant host microorganisms or animals are provided. Pharmaceutical compositions, infant formulas or dietary supplements containing fatty acids which have been desaturated by a  $\Delta 5$ -desaturase produced by a recombinant host microorganism or animal also are described.



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## ALTERED FATTY ACID BIOSYNTHESIS IN INSECT CELLS USING DELTA FIVE DESATURASE

**INTRODUCTION****Field of the Invention**

- 5           This invention relates to modulating levels of enzymes and/or enzyme components relating to production of long chain poly-unsaturated fatty acids (PUFAs) and  $\Delta 5$  desaturases in insects or animals.

**Background**

- 10           Two main families of polyunsaturated fatty acids (PUFAs) are the  $\omega 3$  fatty acids, exemplified by eicosapentaenoic acid (EPA), and the  $\omega 6$  fatty acids, exemplified by arachidonic acid (ARA). PUFAs are important components of the plasma membrane of the cell, where they may be found in such forms as phospholipids. PUFAs are necessary for proper development, particularly in the  
15           developing infant brain, and for tissue formation and repair. PUFAs also serve as precursors to other molecules of importance in human beings and animals, including the prostacyclins, eicosanoids, leukotrienes and prostaglandins.

- Four major long chain PUFAs of importance include docosahexaenoic acid (DHA) and EPA, which are primarily found in different types of fish oil,  
20           gamma-linolenic acid (GLA), which is found in the seeds of a number of plants, including evening primrose (*Oenothera biennis*), borage (*Borago officinalis*) and black currants (*Ribes nigrum*), and stearidonic acid (SDA), which is found in marine oils and plant seeds. Both GLA and another important long chain PUFA, arachidonic acid (ARA), are found in filamentous fungi. ARA can be  
25           purified from animal tissues including liver and adrenal gland. GLA, ARA, EPA and SDA are themselves, or are dietary precursors to, important long chain fatty acids involved in prostaglandin synthesis, in treatment of heart disease, and in development of brain tissue.

Polyunsaturated fatty acids have a number of pharmaceutical and medical applications including treatment of heart disease, cancer and arthritis.

For DHA, a number of sources exist for commercial production including a variety of marine organisms, oils obtained from cold water marine fish, and egg yolk fractions. For ARA, microorganisms including the genera *Mortierella*, *Entomophthora*, *Phytium* and *Porphyridium* can be used for commercial production. Commercial sources of SDA include the genera *Trichodesma* and *Echium*. Commercial sources of GLA include evening primrose, black currants and borage. However, there are several disadvantages associated with commercial production of PUFAs from natural sources. Natural sources of PUFAs, such as animals and plants, tend to have highly heterogeneous oil compositions. The oils obtained from these sources therefore can require extensive purification to separate out one or more desired PUFAs or to produce an oil which is enriched in one or more PUFA. Natural sources also are subject to uncontrollable fluctuations in availability. Fish stocks may undergo natural variation or may be depleted by overfishing. Fish oils have unpleasant tastes and odors, which may be impossible to economically separate from the desired product, and can render such products unacceptable as food supplements. Animal oils, and particularly fish oils, can accumulate environmental pollutants. Weather and disease can cause fluctuation in yields from both fish and plant sources. Cropland available for production of alternate oil-producing crops is subject to competition from the steady expansion of human populations and the associated increased need for food production on the remaining arable land. Crops which do produce PUFAs, such as borage, have not been adapted to commercial growth and may not perform well in monoculture. Growth of such crops is thus not economically competitive where more profitable and better established crops can be grown. Large scale fermentation of organisms such as *Mortierella* is also expensive. Natural animal tissues contain low amounts of ARA and are difficult to process. Microorganisms such as *Porphyridium* and *Mortierella* are difficult to cultivate on a commercial scale.



Dietary supplements and pharmaceutical formulations containing PUFAs can retain the disadvantages of the PUFA source. Supplements such as fish oil capsules can contain low levels of the particular desired component and thus require large dosages. High dosages result in ingestion of high levels of undesired components, including contaminants. Unpleasant tastes and odors of the supplements can make such regimens undesirable, and may inhibit compliance by the patient. Care must be taken in providing fatty acid supplements, as overaddition may result in suppression of endogenous biosynthetic pathways and lead to competition with other necessary fatty acids in various lipid fractions *in vivo*, leading to undesirable results. For example, Eskimos having a diet high in  $\omega$ 3 fatty acids have an increased tendency to bleed (U.S. Pat. No. 4,874,603).

A number of enzymes are involved in PUFA biosynthesis. Linolenic acid (LA, 18:2  $\Delta$ 9, 12) is produced from oleic acid (18:1  $\Delta$ 9) by a  $\Delta$ 12-desaturase. GLA (18:3  $\Delta$ 6, 9, 12) is produced from linoleic acid (LA, 18:2  $\Delta$ 9, 12) by a  $\Delta$ 6-desaturase. ARA (20:4  $\Delta$ 5, 8, 11, 14) production from dihomo-gamma-linolenic acid (DGLA, 20:3  $\Delta$ 8, 11, 14) is catalyzed by a  $\Delta$ 5-desaturase. However, animals cannot desaturate beyond the  $\Delta$ 9 position and therefore cannot convert oleic acid (18:1  $\Delta$ 9) into linolenic acid (18:2  $\Delta$ 9, 12). Likewise,  $\alpha$ -linoleic acid (ALA, 18:3  $\Delta$ 9, 12, 15) cannot be synthesized by mammals. Other eukaryotes, including fungi and plants, have enzymes which desaturate at positions  $\Delta$ 12 and  $\Delta$ 15. The major poly-unsaturated fatty acids of animals therefore are either derived from diet and/or from desaturation and elongation of linoleic acid (18:2  $\Delta$ 9, 12) or  $\alpha$ -linolenic acid (18:3  $\Delta$ 9, 12, 15). Therefore it is of interest to obtain genetic material involved in PUFA biosynthesis from species that naturally produce these fatty acids and to express the isolated material in a microbial or animal system which can be manipulated to provide production of commercial quantities of one or more PUFAs. Thus there is a need for fatty acid desaturases, genes encoding them, and recombinant methods of producing them. A need further exists for oils containing higher relative

proportions of and/or enriched in specific PUFAs. A need also exists for reliable economical methods of producing specific PUFAs.

In particular, there is a need to express PUFAs in a baculovirus expression system using cultured insect cells such as *Spodoptera frugiperda* (Sf9), army fallworm. The lipid composition of Sf9 cells has been well characterized and are known to contain low proportions of polyunsaturated fatty acids. As such, production of PUFAs in insect cells would provide a good source of purified  $\Delta 5$  desaturases for use in enzyme studies and antibody production as well as a good source of PUFAs for use in various oils, nutritional supplements, cosmetic agents and the like.

### Summary of the Invention

Novel compositions and methods are provided for preparation of polyunsaturated long chain fatty acids or PUFAs. The compositions include nucleic acids encoding a  $\Delta 5$ -desaturase and/or polypeptides having  $\Delta 5$ -desaturase activity, the polypeptides, and probes for isolating and detecting the same. The methods involve growing a host microorganism or animal which contains and expresses one or more transgenes encoding a  $\Delta 5$ -desaturase and/or a polypeptide having  $\Delta 5$ -desaturase activity. Expression of the desaturase polypeptide provides for a relative increase in  $\Delta 5$ -desaturated PUFA, or metabolic progeny therefrom, as a result of altered concentrations of enzymes and substrates involved in PUFA biosynthesis. The invention finds use for example in the large scale production of PUFA containing oils which include, for example, ARA, EPA and/or DHA.

The invention is also directed to methods of producing purified  $\Delta 5$  desaturase in an insect cell culture system. The purified protein can be used as an antigen for the production of polyclonal and monoclonal antibodies. These antibodies find use in methods of purifying the  $\Delta 5$  desaturases and in methods of detecting levels of  $\Delta 5$  desaturase polypeptide in complex solutions.

The purified  $\Delta 5$  desaturase protein also finds use in studies of the  $\Delta 5$  desaturase enzyme including X-ray crystal structures for structure-function studies. Once the purified protein has been studied in some detail, the enzyme can be modified by genetic engineering techniques to improve enzymatic activity and to alter substrate specificities.

In a preferred embodiment, a nucleic acid sequence comprising a  $\Delta 5$ -desaturase depicted in Figure 3A-D (SEQ ID NO 1), a polypeptide encoded by the nucleic acid, and a purified or isolated polypeptide depicted in Figure 3A-D (SEQ ID NO: 2), and an isolated nucleic acid encoding the polypeptide of Figure 3A-D (SEQ ID NO: 2) are provided. Another embodiment of the invention is an isolated nucleic acid sequence which encodes a polypeptide, wherein said polypeptide desaturates a fatty acid molecule at carbon 5 from the carboxyl end of the molecule. The nucleic acid is preferably derived from a eukaryotic cell, such as a fungal cell, or a fungal cell of the genus *Mortierella*, or of the genus/species *Mortierella alpina*. Also preferred is an isolated nucleic acid comprising a sequence which anneals to a nucleotide sequence depicted in Figure 3A-3D (SEQ ID NO: 1), and a nucleic acid which encodes an amino acid sequence depicted in Figure 3A-D (SEQ ID NO: 2). In particular, the nucleic acid encodes an amino acid sequence depicted in Figure 3A-D (SEQ ID NO: 2) which is selected from the group consisting of amino acid residues 30-38, 41-44, 171-175, 203-212, and 387-394. In an additional embodiment, the invention provides an isolated or purified polypeptide which desaturates a fatty acid molecule at carbon 5 from the carboxyl end of the molecule. Also provided is an isolated nucleic acid sequence which hybridizes to a nucleotide sequence depicted in Figure 3A-D (SEQ ID NO 1), an isolated nucleic acid sequence having at least about 50% identity to Figure 3A-D (SEQ ID NO 1).

The present invention further includes a nucleic acid construct comprising a nucleotide sequence depicted in a Figure 3A-D (SEQ ID NO: 1) linked to a heterologous nucleic acid; a nucleic acid construct comprising a nucleotide sequence depicted in a Figure 3A-D (SEQ ID NO: 1) operably linked to a promoter; and a nucleic acid construct comprising a nucleotide sequence

depicted in a Figure 3A-D (SEQ ID NO: 1) operably linked to a promoter which is functional in a microbial cell. In a preferred embodiment, the microbial cell is a yeast cell, and the nucleotide sequence is derived from a fungus, such as a fungus of the genus *Mortierella*, particularly a fungus of the species *Mortierella alpina*.

In another embodiment of the invention, a nucleic acid construct is provided which comprises a nucleotide sequence which encodes a polypeptide comprising an amino acid sequence which corresponds to or is complementary to an amino acid sequence depicted in Figure 3A-D (SEQ ID NO: 2), wherein the nucleotide sequence is operably linked to a promoter which is functional in a host cell, and wherein the nucleotide sequence encodes a polypeptide which desaturates a fatty acid molecule at carbon 5 from the carboxyl end of a fatty acid molecule. Additionally, provided by the invention is a nucleic acid construct comprising a nucleotide sequence which encodes a functionally active  $\Delta 5$ -desaturase, where the desaturase includes an amino acid sequence which corresponds to or is complementary to all of or a portion of an amino acid sequence depicted in a Figure 3A-D (SEQ ID NO: 2), wherein the nucleotide sequence is operably linked to a promoter functional in a host cell.

The invention also includes a host cell comprising a nucleic acid construct of the invention. In a preferred embodiment, a recombinant host cell is provided which comprises at least one copy of a DNA sequence which encodes a functionally active *Mortierella alpina* fatty acid desaturase having an amino acid sequence as depicted in Figure 3A-D (SEQ ID NO: 2), wherein the cell or an ancestor of the cell was transformed with a vector comprising said DNA sequence, and wherein the DNA sequence is operably linked to a promoter. The host cell is either eukaryotic or prokaryotic. Preferred eukaryotic host cells are those selected from the group consisting of a mammalian cell, an insect cell, a fungal cell, and an algae cell. A preferred algae is a marine algae. Most preferred is an insect cell. Most preferred insect cells include fall army worm cells, *Spodoptera frugiperda* (Sf9 and Sf21 cells) and cabbage looper moth, *Trichoplusia ni* (Hi Five Cells).

Preferred prokaryotic cells include those selected from the group consisting of a bacteria, a cyanobacteria, cells which contain a bacteriophage, and/or a virus. The DNA sequence of the recombinant host cell preferably contains a promoter which is functional in the host cell.

5           The host cells of the invention which contain the DNA sequences of the invention are enriched for fatty acids, such as 20:3 fatty acids. In a preferred embodiment, the host cells are enriched for 20:4 fatty acids as compared to an untransformed host cell which is devoid of said DNA sequence, and/or enriched for 20:5 fatty acids compared to an untransformed host cell which is devoid of  
10       said DNA sequence. In yet another preferred embodiment, the invention provides a recombinant host cell which comprises a fatty acid selected from the group consisting of a dihomo- $\gamma$ -linolenic acid, n-6 eicosatrienoic acid, 20:3n-6 acid and 20:3 (8,11,14) acid.

          The present invention also includes method for production of  
15       arachidonic acid in a insect cell culture, where the method comprises growing an insect cell culture having a plurality of insect cells which contain one or more nucleic acids encoding a polypeptide which converts dihomo- $\gamma$ -linolenic acid to arachidonic acid, wherein the nucleic acid is operably linked to a promoter, under conditions whereby said one or more nucleic acids are  
20       expressed, whereby arachidonic acid is produced in the microbial cell culture. In several preferred embodiments of the invention, the polypeptide is an enzyme which desaturates a fatty acid molecule at carbon 5 from the carboxyl end of the fatty acid molecule; the nucleic acid is derived from a *Mortierella sp.*; and the substrate for said polypeptide is exogenously supplied. Preferred insect cells  
25       include *spodoptera frugiper*a (Sf9 and Sf 21). Preferred mammalian cells include an avian cell, a preferred fungal cell is a yeast, and the preferred algae cell is a marine algae cell. The preferred prokaryotic cells include those selected from the group consisting of a bacteria, a cyanobacteria, cells which contain a bacteriophage, and/or a virus. The nucleic acid sequence encoding  
30       the polypeptide of the microbial cell preferably contains a promoter which is functional in the host cell which optionally is an inducible promoter for example

by components of the culture broth. The preferred microbial cells used in the methods are yeast cells, such as *Saccharomyces* cells.

In another embodiment of the invention, a recombinant yeast cell is provided which converts greater than about 5% of 20:3 fatty acid substrate to a  
5 20:4 fatty acid product.

Also provided is an oil comprising one or more PUFA. The amount of said one or more PUFAs is approximately 0.3-30% arachidonic acid (ARA), approximately 0.2-30% dihomo- $\gamma$ -linolenic acid (DGLA), and approximately 0.2-30%  $\gamma$ -linolenic acid (GLA). A preferred oil of the invention is one in  
10 which the ratio of ARA:DGLA:GLA is approximately 1.0:19.0:30 to 6.0:1.0:0.2. Another preferred embodiment of the invention is a pharmaceutical composition comprising the oils in a pharmaceutically acceptable carrier. Further provided is a nutritional composition comprising the oils of the invention. The nutritional compositions of the invention preferably are  
15 administered to a mammalian host parenterally or internally. A preferred composition of the invention for internal consumption is an infant formula. In a preferred embodiment, the nutritional compositions of the invention are in a liquid form or a solid form.

The present invention also includes a method for desaturating a fatty  
20 acid, where the method comprises culturing a recombinant microbial cell of the invention under conditions suitable for expression of a polypeptide encoded by the nucleic acid, wherein the host cell further comprises a fatty acid substrate of the polypeptide. In a preferred embodiment, a fatty acid desaturated by the methods is provided, including an oil comprising the fatty acid.

25 The present invention is also directed to purified nucleotide and peptide sequences presented in SEQ ID NO:1-34. The present invention is further directed toward methods of using the sequences presented in SEQ ID NO:1-34 as probes to identify related sequences, as components of expression systems and as components of systems useful for producing transgenic oil.

The present invention is further directed to methods of obtaining altered long chain poly unsaturated fatty acid biosystems by growing transgenic microbes which encode transgene expression products which desaturate a fatty acid molecule at carbon 5 from the carboxyl end of the fatty acid molecule.

- 5           The present invention is further directed to formulas, dietary supplements or dietary substitutes in the form of a liquid or a solid containing the long chain fatty acids of the invention. These formulas, supplements and substitutes may be administered to a human or an animal.

- 10           The formulas, supplements of the invention may further comprise at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electro dialysed whey, electro dialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

- 15           The formulas of the present invention may further include at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.

- 20           The present invention is further directed to a method of treating a patient having a condition caused by insufficient intake or production of polyunsaturated fatty acids comprising administering to the patient a dietary substitute of the invention in an amount sufficient to effect treatment of the patient.

- 25           The present invention is further directed to cosmetic and pharmaceutical compositions of the material of the invention.

- 30           The present invention is also directed to an isolated nucleotide sequence comprising a nucleotide sequence selected from the group consisting of: SEQ ID NO:13; SEQ ID NO:15; SEQ ID NO:17; SEQ ID NO:19; SEQ ID NO:21; SEQ ID NO:22; SEQ ID NO:22; SEQ ID NO:24; SEQ ID NO:25; SEQ ID NO:26 and SEQ ID NO:27.

The present invention is also directed to an isolated peptide sequence comprising a peptide sequence selected from the group consisting of: SEQ ID NO:14; SEQ ID NO:16; SEQ ID NO:18; SEQ ID NO:20; SEQ ID NO:28; SEQ ID NO:29; SEQ ID NO:30; SEQ ID NO:31; SEQ ID NO:32; SEQ ID  
5 NO:33 and SEQ ID NO:34.

The present invention is further directed to transgenic oils in pharmaceutically acceptable carriers. The present invention is further directed to nutritional supplements, cosmetic agents and infant formulae containing transgenic oils.

10 The present invention is further directed to a method for obtaining altered long chain polyunsaturated fatty acid biosynthesis comprising the steps of: growing a microbe having cells which contain a transgene which encodes a transgene expression product which desaturates a fatty acid molecule at carbon  
5 from the carboxyl end of said fatty acid molecule, wherein the transgene is  
15 operably associated with an expression control sequence, under conditions whereby the transgene is expressed, whereby long chain polyunsaturated fatty acid biosynthesis in the cells is altered.

The present invention is further directed to the use of chain polyunsaturated fatty acid selected from the group consisting of ARA, DGLA  
20 and EPA.

\_\_\_ The present invention is further directed toward pharmaceutical compositions comprising at least one nutrient selected from the group consisting of a vitamin, a mineral, a carbohydrate, a sugar, an amino acid, a free fatty acid, a phospholipid, an antioxidant, and a phenolic compound.



### Brief Description of the Drawings

Figure 1 shows possible pathways for the synthesis of arachidonic acid (20:4  $\Delta$ 5, 8, 11, 14) and stearidonic acid (18:4  $\Delta$ 6, 9, 12, 15) from palmitic acid ( $C_{16}$ ) from a variety of organisms, including algae, *Mortierella* and humans.

- 5 These PUFAs can serve as precursors to other molecules important for humans and other animals, including prostacyclins, leukotrienes, and prostaglandins, some of which are shown.

Figure 2 shows possible pathways for production of PUFAs in addition to ARA, including EPA and DHA, for a variety of organisms.

- 10 Figure 3A-D shows the DNA sequence of the *Mortierella alpina*  $\Delta$ 5-desaturase and the deduced amino acid sequence.

Figure 4 shows the deduced amino acid sequence of the PCR fragment (see Example 1)

- 15 Figure 5A and 5B show alignments of the protein sequence of the  $\Delta$ 5-desaturase with  $\Delta$ 6-desaturases.

Figure 6A and 6B show the effect of the timing of substrate addition relative to induction on conversion of substrate to product in SC334 containing the  $\Delta$ 5-desaturase gene.

- 20 Figure 7A and 7B show the effect of inducer concentration on  $\Delta$ 5-desaturase expression in SC334.

Figure 8A and 8B show the effect of induction temperature on  $\Delta$ 5-desaturase activity in SC334.

Figure 9A and 9B show the effect of host strain on the conversion of substrate to product in strains expressing the  $\Delta$ 5-desaturase gene at 15°C.

- 25 Figure 10A and 10B show the effect of host strain on the conversion of substrate to product in strains expressing the  $\Delta$ 5-desaturase gene at 30°C.

Figure 11 shows the effect of a host strain expressing choline transferase as well as the  $\Delta 5$ -desaturase gene on the conversion of substrate to product.

Figure 12A and 12B show the effect of media composition and temperature on the conversion of substrate to product in two host strains  
5 expressing the  $\Delta 5$ -desaturase gene.

Figure 13 shows alignment of the protein sequence of Ma 29 and contig 253538a.

Figure 14 shows alignment of the protein sequence of Ma 524 and contig 253538a.

10

### Brief Description of the Sequence Listings

SEQ ID NO:1 shows a DNA sequence of the *Mortierella alpina*  $\Delta 5$ -desaturase.

SEQ ID NO:2 shows an amino acid sequence of *Mortierella alpina*  $\Delta 5$ -  
15 desaturase.

SEQ ID NO: 3 shows the deduced amino acid sequence of the *M. alpina* PCR fragment (see Example 1).

SEQ ID NO: 4 - SEQ ID NO: 7 show the deduced amino acid sequences of various  $\Delta 6$ -desaturases.

20 SEQ ID NO: 8 and SEQ ID NO: 9 show PCR primer sequences for  $\Delta 6$ -desaturases

SEQ ID NO: 10 shows a primer for reverse transcription of total RNA.

SEQ ID NO: 11 and SEQ ID NO: 12 show amino acid motifs for desaturase sequences.

25 SEQ ID NO: 13 and SEQ ID NO: 14 show the nucleotide and amino acid sequence of a *Dictyostelium discoideum* desaturase sequence.

SEQ ID NO: 15 and SEQ ID NO: 16 show the nucleotide and amino acid sequence of a *Phaeodactylum tricornutum* desaturase sequence.

SEQ ID NO: 17-20 show the nucleotide and deduced amino acid sequence of a *Schizochytrium* cDNA clone.

5 SEQ ID NO: 21-27 show nucleotide sequences for human desaturases.

SEQ ID NO: 28 - SEQ ID NO: 34 show peptide sequences for human desaturases.

### Detailed Description of the Invention

10 In order to ensure a complete understanding of the invention, the following definitions are provided:

**$\Delta$ 5-Desaturase:**  $\Delta$ 5 desaturase is an enzyme which introduces a double bond between carbons 5 and 6 from the carboxyl end of a fatty acid molecule.

15  **$\Delta$ 6-Desaturase:**  $\Delta$ 6-desaturase is an enzyme which introduces a double bond between carbons 6 and 7 from the carboxyl end of a fatty acid molecule.

**$\Delta$ 9-Desaturase:**  $\Delta$ 9-desaturase is an enzyme which introduces a double bond between carbons 9 and 10 from the carboxyl end of a fatty acid molecule.

20  **$\Delta$ 12-Desaturase:**  $\Delta$ 12-desaturase is an enzyme which introduces a double bond between carbons 12 and 13 from the carboxyl end of a fatty acid molecule.

**Fatty Acids:** Fatty acids are a class of compounds containing a long hydrocarbon chain and a terminal carboxylate group. Fatty acids include the following:

| Fatty Acid |                  |  |
|------------|------------------|--|
| 12:0       | lauric acid      |  |
| 16:0       | palmitic acid    |  |
| 16:1       | palmitoleic acid |  |

| Fatty Acid           |  |   |
|----------------------|--|---|
| 18:0                 | stearic acid   |   |
| 18:1                 | oleic acid   | $\Delta 9-18:1$                               |
| 18:2 $\Delta 5,9$    | taxoleic acid  | $\Delta 5,9-18:2$                             |
| 18:2 $\Delta 6,9$    | 6,9-octadecadienoic acid                             | $\Delta 6,9-18:2$                             |
| 18:2                 | linoleic acid  | $\Delta 9,12-18:2$ (LA)                       |
| 18:3 $\Delta 6,9,12$ | gamma-linolenic acid                                 | $\Delta 6,9,12-18:3$ (GLA)                    |
| 18:3 $\Delta 5,9,12$ | pinolenic acid                                       | $\Delta 5,9,12-18:3$                          |
| 18:3                 | alpha-linolenic acid                                 | $\Delta 9,12,15-18:3$ (ALA)                   |
| 18:4                 | stearidonic acid                                     | $\Delta 6,9,12,15-18:4$ (SDA)                 |
| 20:0                 | Arachidic acid                                       |   |
| 20:1                 | Eicosenic Acid                                       |   |
| 22:0                 | behehic acid   |   |
| 22:1                 | erucic acid  |   |
| 22:2                 | Docasadienoic acid                                   |   |
| 20:4 $\omega 6$      | arachidonic acid                                     | $\Delta 5,8,11,14-20:4$ (ARA)                 |
| 20:3 $\omega 6$      | $\omega 6$ -eicosatrienoic<br>dihomo-gamma linolenic | $\Delta 8,11,14-20:3$ (DGLA)                  |
| 20:5 $\omega 3$      | Eicosapentanoic<br>(Timnodonic acid)                 | $\Delta 5,8,11,14,17-20:5$ (EPA)              |
| 20:3 $\omega 3$      | $\omega 3$ -eicosatrienoic                           | $\Delta 11,16,17-20:3$                        |
| 20:4 $\omega 3$      | $\omega 3$ -eicosatetraenoic                         | $\Delta 8,11,14,17-20:4$                      |
| 22:5 $\omega 3$      | Docasapentaenoic                                     | $\Delta 7,10,13,16,19-22:5$ ( $\omega 3$ DPA) |
| 22:6 $\omega 3$      | Docosahexaenoic<br>(cervonic acid)                   | $\Delta 4,7,10,13,16,19-22:6$ (DHA)           |
| 24:0                 | Lignoceric acid                                      |   |

5 Taking into account these definitions, the present invention is directed to novel DNA sequences, DNA constructs, methods and compositions are provided which permit modification of the poly-unsaturated long chain fatty acid content of, for example, microbial cells or animals. Host cells are manipulated to express a sense or antisense transcript of a DNA encoding a polypeptide(s) which catalyzes the conversion of DGLA to ARA. The substrate(s) for the expressed enzyme may be produced by the host cell or may be exogenously supplied. To achieve expression, the transformed DNA is

operably associated with transcriptional and translational initiation and termination regulatory regions that are functional in the host cell. Constructs comprising the gene to be expressed can provide for integration into the genome of the host cell or can autonomously replicate in the host cell. For production of ARA, the expression cassettes generally used include a cassette which provides for  $\Delta 5$ -desaturase activity, particularly in a host cell which produces or can take up DGLA. Production of  $\omega 6$ -type unsaturated fatty acids, such as ARA, is favored in a host microorganism or animal which is substantially free of ALA. The host is selected or obtained by removing or inhibiting activity of a  $\Delta 15$  desaturase or a desaturase involved in the omega-3 pathway (see Figure 2). The endogenous desaturase activity can be affected by providing an expression cassette for an antisense  $\Delta 15$  or  $\omega 3$  transcript, by disrupting a target  $\Delta 15$ - or  $\omega 3$ -desaturase gene through insertion, substitution and/or deletion of all or part of the target gene, or by adding a  $\Delta 15$ - or  $\omega 3$ -desaturase inhibitor. Production of LA also can be increased by providing expression cassettes for  $\Delta 9$  and/or  $\Delta 12$ -desaturases where their respective enzymatic activities are limiting.

### INSECT CELL PRODUCTION OF FATTY ACIDS

Insect cell production of fatty acids has several advantages over purification from natural sources such as fish or plants. Many insect cells are known with greatly simplified lipid compositions compared with those of higher organisms, making purification of desired components easier. Insect cell fatty acid production is not subject to fluctuations caused by external variables such as weather and food supply. Insect cell produced fatty acids are substantially free of contamination by environmental pollutants. Additionally, insect cells can provide PUFAs in particular forms which may have specific uses. Additionally, insect cell lipid production can be manipulated by controlling culture conditions, notably by providing particular substrates for microbially expressed enzymes, or by addition of compounds which suppress undesired biochemical pathways. In addition to these advantages, production of fatty

acids from recombinant microbes provides the ability to alter the naturally occurring microbial fatty acid profile by providing new synthetic pathways in the host or by suppressing undesired pathways, thereby increasing levels of desired PUFAs, or conjugated forms thereof, and decreasing levels of undesired PUFAs.

### PRODUCTION OF FATTY ACIDS IN ANIMALS

Production of fatty acids in animals also presents several advantages. Expression of desaturase genes in animals can produce greatly increased levels of desired PUFAs in animal tissues, making recovery from those tissues more economical. For example, where the desired PUFAs are expressed in the breast milk of animals, methods of isolating PUFAs from animal milk are well established. In addition to providing a source for purification of desired PUFAs, animal breast milk can be manipulated through expression of desaturase genes, either alone or in combination with other human genes, to provide animal milks with a PUFA composition substantially similar to human breast milk during the different stages of infant development. Humanized animal milks could serve as infant formulas where human nursing is impossible or undesired, or in cases of malnourishment or disease.

Depending upon the host cell, the availability of substrate, and the desired end product(s), several polypeptides, particularly desaturases, are of interest. By "desaturase" is intended a polypeptide which can desaturate one or more fatty acids to produce a mono- or poly-unsaturated fatty acid or precursor thereof of interest. Of particular interest are polypeptides which can catalyze the conversion of DGLA to produce ARA which includes enzymes which desaturate at the  $\Delta 5$  position. By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification, for example, glycosylation or phosphorylation. Considerations for choosing a specific polypeptide having desaturase activity include the pH optimum of the polypeptide, whether the polypeptide is a rate limiting enzyme or a component thereof, whether the desaturase used is essential for synthesis of a desired poly-unsaturated fatty acid, and/or co-factors required by the polypeptide. The

expressed polypeptide preferably has parameters compatible with the biochemical environment of its location in the host cell. For example, the polypeptide may have to compete for substrate with other enzymes in the host cell. Analyses of the  $K_m$  and specific activity of the polypeptide in question  
5 therefore are considered in determining the suitability of a given polypeptide for modifying PUFA production in a given host cell. The polypeptide used in a particular situation is one which can function under the conditions present in the intended host cell but otherwise can be any polypeptide having desaturase activity which has the desired characteristic of being capable of modifying the  
10 relative production of a desired PUFA.

For production of ARA, the DNA sequence used encodes a polypeptide having  $\Delta 5$ -desaturase activity. In particular instances, this can be coupled with an expression cassette which provides for production of a polypeptide having  $\Delta 6$ -desaturase activity and the host cell can optionally be depleted of any  $\Delta 15$ -  
15 desaturase activity present, for example by providing a transcription cassette for production of antisense sequences to the  $\Delta 15$ -desaturase transcription product, by disrupting the  $\Delta 15$ -desaturase gene, or by using a host cell which naturally has, or has been mutated to have, low  $\Delta 15$ -desaturase activity. Inhibition of undesired desaturase pathways also can be accomplished through the use of  
20 specific desaturase inhibitors such as those described in U.S. Patent No. 4,778,630. The choice of combination of cassettes used can depend in part on the PUFA profile of the host cell. Where the host cell  $\Delta 5$ -desaturase activity is limiting, overexpression of  $\Delta 5$ -desaturase alone generally will be sufficient to provide for enhanced ARA production in the presence of an appropriate  
25 substrate such as DGLA. ARA production also can be increased by providing expression cassettes for  $\Delta 9$ - or  $\Delta 12$ -desaturase genes when the activities of those desaturases are limiting. A scheme for the synthesis of arachidonic acid ( $20:4 \Delta^{5, 8, 11, 14}$ ) from palmitic acid ( $C_{16}$ ) is shown in Figure 1. A key enzyme in this pathway is a  $\Delta 5$ -desaturase which converts DH- $\gamma$ -linolenic acid (DGLA,  
30 eicosatrienoic acid) to ARA. Conversion of  $\alpha$ -linolenic acid (ALA) to

stearidonic acid by a  $\Delta 6$ -desaturase is also shown. Production of PUFAs in addition to ARA, including EPA and DHA is shown in Figure 2.

### SOURCES OF POLYPEPTIDES HAVING DESATURASE ACTIVITY

5           A source of polypeptides having desaturase activity and oligonucleotides encoding such polypeptides are organisms which produce a desired poly-unsaturated fatty acid. As an example, microorganisms having an ability to produce ARA can be used as a source of  $\Delta 5$ -desaturase activity. Such microorganisms include, for example, those belonging to the genera  
10   *Mortierella*, *Conidiobolus*, *Pythium*, *Phytophthora*, *Penicillium*, *Porphyridium*, *Coidosporium*, *Mucor*, *Fusarium*, *Aspergillus*, *Rhodotorula*, and *Entomophthora*. Within the genus *Porphyridium*, of particular interest is *Porphyridium cruentum*. Within the genus *Mortierella*, of particular interest are *Mortierella elongata*, *Mortierella exigua*, *Mortierella hygrophila*, *Mortierella*  
15   *ramanniana*, var. *angulispora*, and *Mortierella alpina*. Within the genus *Mucor*, of particular interest are *Mucor circinelloides* and *Mucor javanicus*.

          DNAs encoding desired desaturases can be identified in a variety of ways. As an example, a source of the desired desaturase, for example genomic or cDNA libraries from *Mortierella*, is screened with detectable enzymatically-  
20   or chemically-synthesized probes, which can be made from DNA, RNA, or non-naturally occurring nucleotides, or mixtures thereof. Probes may be enzymatically synthesized from DNAs of known desaturases for normal or reduced-stringency hybridization methods. Oligonucleotide probes also can be used to screen sources and can be based on sequences of known desaturases,  
25   including sequences conserved among known desaturases, or on peptide sequences obtained from the desired purified protein. Oligonucleotide probes based on amino acid sequences can be degenerate to encompass the degeneracy of the genetic code, or can be biased in favor of the preferred codons of the source organism. Oligonucleotides also can be used as primers for PCR from  
30   reverse transcribed mRNA from a known or suspected source; the PCR product can be the full length cDNA or can be used to generate a probe to obtain the



desired full length cDNA. Alternatively, a desired protein can be entirely sequenced and total synthesis of a DNA encoding that polypeptide performed.

Once the desired genomic or cDNA has been isolated, it can be sequenced by known methods. It is recognized in the art that such methods are subject to errors, such that multiple sequencing of the same region is routine and is still expected to lead to measurable rates of mistakes in the resulting deduced sequence, particularly in regions having repeated domains, extensive secondary structure, or unusual base compositions, such as regions with high GC base content. When discrepancies arise, resequencing can be done and can employ special methods. Special methods can include altering sequencing conditions by using: different temperatures; different enzymes; proteins which alter the ability of oligonucleotides to form higher order structures; altered nucleotides such as ITP or methylated dGTP; different gel compositions, for example adding formamide; different primers or primers located at different distances from the problem region; or different templates such as single stranded DNAs. Sequencing of mRNA also can be employed.

For the most part, some or all of the coding sequence for the polypeptide having desaturase activity is from a natural source. In some situations, however, it is desirable to modify all or a portion of the codons, for example, to enhance expression, by employing host preferred codons. Host preferred codons can be determined from the codons of highest frequency in the proteins expressed in the largest amount in a particular host species of interest. Thus, the coding sequence for a polypeptide having desaturase activity can be synthesized in whole or in part. All or portions of the DNA also can be synthesized to remove any destabilizing sequences or regions of secondary structure which would be present in the transcribed mRNA. All or portions of the DNA also can be synthesized to alter the base composition to one more preferable in the desired host cell. Methods for synthesizing sequences and bringing sequences together are well established in the literature. *In vitro* mutagenesis and selection, site-directed mutagenesis, or other means can be employed to obtain mutations of naturally occurring desaturase genes to

produce a polypeptide having desaturase activity *in vivo* with more desirable physical and kinetic parameters for function in the host cell, such as a longer half-life or a higher rate of production of a desired polyunsaturated fatty acid.

**Mortierella alpina Desaturase**

5           Of particular interest is the *Mortierella alpina*  $\Delta 5$ -desaturase which has 446 amino acids; the amino acid sequence is shown in Figure 3. The gene encoding the *Mortierella alpina*  $\Delta 5$ -desaturase can be expressed in transgenic microorganisms or animals to effect greater synthesis of ARA from DGLA. Other DNAs which are substantially identical to the *Mortierella alpina*  $\Delta 5$ -  
10   desaturase DNA, or which encode polypeptides which are substantially identical to the *Mortierella alpina*  $\Delta 5$ -desaturase polypeptide, also can be used. By substantially identical is intended an amino acid sequence or nucleic acid sequence exhibiting in order of increasing preference at least 60%, 80%, 90% or  
15   95% homology to the *Mortierella alpina*  $\Delta 5$ -desaturase amino acid sequence or nucleic acid sequence encoding the amino acid sequence. For polypeptides, the length of comparison sequences generally is at least 16 amino acids, preferably at least 20 amino acids, or most preferably 35 amino acids. For nucleic acids, the length of comparison sequences generally is at least 50 nucleotides, preferably at least 60 nucleotides, and more preferably at least 75 nucleotides,  
20   and most preferably, 110 nucleotides. Homology typically is measured using sequence analysis software, for example, the Sequence Analysis software package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin 53705, MEGAlign (DNASar, Inc., 1228 S. Park St., Madison, Wisconsin 53715), and  
25   MacVector (Oxford Molecular Group, 2105 S. Bascom Avenue, Suite 200, Campbell, California 95008). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine and leucine;  
30   aspartic acid, glutamic acid, asparagine, and glutamine; serine and threonine;

lysine and arginine; and phenylalanine and tyrosine. Substitutions may also be made on the basis of conserved hydrophobicity or hydrophilicity (Kyte and Doolittle, *J. Mol. Biol.* 157: 105-132, 1982), or on the basis of the ability to assume similar polypeptide secondary structure (Chou and Fasman, *Adv. Enzymol.* 47: 45-148, 1978).

### Other Desaturases

Encompassed by the present invention are related desaturases from the same or other organisms. Such related desaturases include variants of the disclosed  $\Delta 5$ -desaturase naturally occurring within the same or different species of *Mortierella*, as well as homologues of the disclosed  $\Delta 5$ -desaturase from other species. Also included are desaturases which, although not substantially identical to the *Mortierella alpina*  $\Delta 5$ -desaturase, desaturate a fatty acid molecule at carbon 5 from the carboxyl end of a fatty acid molecule. Related desaturases can be identified by their ability to function substantially the same as the disclosed desaturases; that is, are still able to effectively convert DGLA to ARA. Related desaturases also can be identified by screening sequence databases for sequences homologous to the disclosed desaturase, by hybridization of a probe based on the disclosed desaturase to a library constructed from the source organism, or by RT-PCR using mRNA from the source organism and primers based on the disclosed desaturase. Such desaturases include those from humans, *Dictyostelium discoideum* and *Phaeodactylum tricornum*.

The regions of a desaturase polypeptide important for desaturase activity can be determined through routine mutagenesis, expression of the resulting mutant polypeptides and determination of their activities. Mutants may include deletions, insertions and point mutations, or combinations thereof. A typical functional analysis begins with deletion mutagenesis to determine the N- and C-terminal limits of the protein necessary for function, and then internal deletions, insertions or point mutants are made to further determine regions necessary for function. Other techniques such as cassette mutagenesis or total synthesis also

can be used. Deletion mutagenesis is accomplished, for example, by using exonucleases to sequentially remove the 5' or 3' coding regions. Kits are available for such techniques. After deletion, the coding region is completed by ligating oligonucleotides containing start or stop codons to the deleted coding  
5 region after 5' or 3' deletion, respectively. Alternatively, oligonucleotides encoding start or stop codons are inserted into the coding region by a variety of methods including site-directed mutagenesis, mutagenic PCR or by ligation onto DNA digested at existing restriction sites. Internal deletions can similarly be made through a variety of methods including the use of existing restriction  
10 sites in the DNA, by use of mutagenic primers via site directed mutagenesis or mutagenic PCR. Insertions are made through methods such as linker-scanning mutagenesis, site-directed mutagenesis or mutagenic PCR. Point mutations are made through techniques such as site-directed mutagenesis or mutagenic PCR.

Chemical mutagenesis also can be used for identifying regions of a  
15 desaturase polypeptide important for activity. A mutated construct is expressed, and the ability of the resulting altered protein to function as a desaturase is assayed. Such structure-function analysis can determine which regions may be deleted, which regions tolerate insertions, and which point mutations allow the mutant protein to function in substantially the same way as the native  
20 desaturase. All such mutant proteins and nucleotide sequences encoding them are within the scope of the present invention.

### EXPRESSION OF DESATURASE GENES

Once the DNA encoding a desaturase polypeptide has been obtained, it is placed in a vector capable of replication in a host cell, or is propagated *in*  
25 *vitro* by means of techniques such as PCR or long PCR. Replicating vectors can include plasmids, phage, viruses, cosmids and the like. Desirable vectors include those useful for mutagenesis of the gene of interest or for expression of the gene of interest in host cells. The technique of long PCR has made *in vitro* propagation of large constructs possible, so that modifications to the gene of  
30 interest, such as mutagenesis or addition of expression signals, and propagation

of the resulting constructs can occur entirely *in vitro* without the use of a replicating vector or a host cell.

For expression of a desaturase polypeptide, functional transcriptional and translational initiation and termination regions are operably linked to the DNA encoding the desaturase polypeptide. Expression of the polypeptide coding region can take place *in vitro* or in a host cell. Transcriptional and translational initiation and termination regions are derived from a variety of nonexclusive sources, including the DNA to be expressed, genes known or suspected to be capable of expression in the desired system, expression vectors, chemical synthesis, or from an endogenous locus in a host cell.

### **Expression In Vitro**

*In vitro* expression can be accomplished, for example, by placing the coding region for the desaturase polypeptide in an expression vector designed for *in vitro* use and adding rabbit reticulocyte lysate and cofactors; labeled amino acids can be incorporated if desired. Such *in vitro* expression vectors may provide some or all of the expression signals necessary in the system used. These methods are well known in the art and the components of the system are commercially available. The reaction mixture can then be assayed directly for the polypeptide, for example by determining its activity, or the synthesized polypeptide can be purified and then assayed.

### **Expression In A Host Cell**

Expression in a host cell can be accomplished in a transient or stable fashion. Transient expression can occur from introduced constructs which contain expression signals functional in the host cell, but which constructs do not replicate and rarely integrate in the host cell, or where the host cell is not proliferating. Transient expression also can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, although such inducible systems frequently exhibit a low basal level of expression. Stable expression can be achieved by introduction of a construct

that can integrate into the host genome or that autonomously replicates in the host cell. Stable expression of the gene of interest can be selected for through the use of a selectable marker located on or transfected with the expression construct, followed by selection for cells expressing the marker. When stable  
5 expression results from integration, integration of constructs can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination with the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory  
10 regions can be provided by the endogenous locus.

When increased expression of the desaturase polypeptide in the source organism is desired, several methods can be employed. Additional genes encoding the desaturase polypeptide can be introduced into the host organism. Expression from the native desaturase locus also can be increased through  
15 homologous recombination, for example by inserting a stronger promoter into the host genome to cause increased expression, by removing destabilizing sequences from either the mRNA or the encoded protein by deleting that information from the host genome, or by adding stabilizing sequences to the mRNA (USPN 4,910,141).

20 When it is desirable to express more than one different gene, appropriate regulatory regions and expression methods, introduced genes can be propagated in the host cell through use of replicating vectors or by integration into the host genome. Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of replication.  
25 Each introduced construct, whether integrated or not, should have a different means of selection and should lack homology to the other constructs to maintain stable expression and prevent reassortment of elements among constructs. Judicious choices of regulatory regions, selection means and method of propagation of the introduced construct can be experimentally determined so  
30 that all introduced genes are expressed at the necessary levels to provide for synthesis of the desired products.

## INTRODUCTION OF CONSTRUCTS INTO HOST CELLS

Constructs comprising the gene of interest may be introduced into a host cell by standard techniques. These techniques include transformation, protoplast fusion, lipofection, transfection, transduction, conjugation, infection, bolistic impact, electroporation, microinjection, scraping, or any other method which introduces the gene of interest into the host cell. Methods of transformation which are used include lithium acetate transformation (*Methods in Enzymology*, Vol. 194, p. 186-187, 1991). For convenience, a host cell which has been manipulated by any method to take up a DNA sequence or construct will be referred to as "transformed" or "recombinant" herein.

The subject host will have at least have one copy of the expression construct and may have two or more, depending upon whether the gene is integrated into the genome, amplified, or is present on an extrachromosomal element having multiple copy numbers. Where the subject host is a yeast, four principal types of yeast plasmid vectors can be used: Yeast Integrating plasmids (YIps), Yeast Replicating plasmids (YRps), Yeast Centromere plasmids (YCps), and Yeast Episomal plasmids (YEps). YIps lack a yeast replication origin and must be propagated as integrated elements in the yeast genome. YRps have a chromosomally derived autonomously replicating sequence and are propagated as medium copy number (20 to 40), autonomously replicating, unstably segregating plasmids. YCps have both a replication origin and a centromere sequence and propagate as low copy number (10-20), autonomously replicating, stably segregating plasmids. YEps have an origin of replication from the yeast 2 $\mu$ m plasmid and are propagated as high copy number, autonomously replicating, irregularly segregating plasmids. The presence of the plasmids in yeast can be ensured by maintaining selection for a marker on the plasmid. Of particular interest are the yeast vectors pYES2 (a YEps plasmid available from Invitrogen, confers uracil prototrophy and a GAL1 galactose-inducible promoter for expression), pRS425-pG1 (a YEps plasmid obtained from Dr. T. H. Chang, Ass. Professor of Molecular Genetics, Ohio State University,

containing a constitutive GPD promoter and conferring leucine prototrophy), and pYX424 (a YEp plasmid having a constitutive TP1 promoter and conferring leucine prototrophy; Alber, T. and Kawasaki, G. (1982). *J. Mol. & Appl. Genetics* 1: 419).

- 5           The transformed host cell can be identified by selection for a marker contained on the introduced construct. Alternatively, a separate marker construct may be introduced with the desired construct, as many transformation techniques introduce many DNA molecules into host cells. Typically, transformed hosts are selected for their ability to grow on selective media.
- 10       Selective media may incorporate an antibiotic or lack a factor necessary for growth of the untransformed host, such as a nutrient or growth factor. An introduced marker gene therefor may confer antibiotic resistance, or encode an essential growth factor or enzyme, and permit growth on selective media when expressed in the transformed host. Selection of a transformed host also can
- 15       occur when the expressed marker protein can be detected, either directly or indirectly. The marker protein may be expressed alone or as a fusion to another protein. The marker protein can be detected by its enzymatic activity; for example  $\beta$  galactosidase can convert the substrate X-gal to a colored product, and luciferase can convert luciferin to a light-emitting product. The marker
- 20       protein can be detected by its light-producing or modifying characteristics; for example, the green fluorescent protein of *Aequorea victoria* fluoresces when illuminated with blue light. Antibodies can be used to detect the marker protein or a molecular tag on, for example, a protein of interest. Cells expressing the marker protein or tag can be selected, for example, visually, or
- 25       by techniques such as FACS or panning using antibodies. For selection of yeast transformants, any marker that functions in yeast may be used. Desirably, resistance to kanamycin and the amino glycoside G418 are of interest, as well as ability to grow on media lacking uracil, leucine, lysine or tryptophan.

- 30           The  $\Delta 5$ -desaturase-mediated production of PUFAs can be performed in either prokaryotic or eukaryotic host cells. Prokaryotic cells of interest include *Eschericia*, *Bacillus*, *Lactobacillus*, *cyanobacteria* and the like. Eukaryotic



cells include mammalian cells such as those of lactating animals, avian cells such as of chickens, and other cells amenable to genetic manipulation including insect, fungal, and algae cells. The cells may be cultured or formed as part or all of a host organism including an animal. Viruses and bacteriophage also may be used with the cells in the production of PUFAs, particularly for gene transfer, cellular targeting and selection. In a preferred embodiment, the host is any microorganism or animal which produces DGLA and/or can assimilate exogenously supplied DGLA, and preferably produces large amounts of DGLA. Examples of host animals include mice, rats, rabbits, chickens, quail, turkeys, bovines, sheep, pigs, goats, yaks, etc., which are amenable to genetic manipulation and cloning for rapid expansion of the transgene expressing population. For animals, a  $\Delta 5$ -desaturase transgene can be adapted for expression in target organelles, tissues and body fluids through modification of the gene regulatory regions. Of particular interest is the production of PUFAs in the breast milk of the host animal.

#### Expression In Insect Cells

Production of PUFAs in insect cells can be conducted using baculovirus expression vectors harboring a  $\Delta 5$ -desaturase transgene as detailed in the Example section below. Examples of host systems include *Spodoptera frugiperda*, (Sf9 and Sf21 cells) and cabbage looper moth *Trichoplusia ni* (Hi Five Cells).

Use of the baculovirus expression system in transgenic insect cells offers a number of advantages in PUFA production. Baculovirus expression systems are high level gene expression systems capable of post translational modifications similar to mammalian cells. The expression system allows for genetic manipulation with relative ease in comparison to mammalian cell transfections. By using site-specific transposition and eliminating the potential mix of parental and nonrecombinant baculoviruses, there is no need for multiple rounds of plaque purification. This greatly reduces the time it takes to identify and purify a recombinant virus. This system also offers the advantage of

supporting coexpression of gene products encoded by different recombinant baculovirus vectors in a single infection.

### Expression in Algae Cells

Methods for producing hybrid and transgenic strains of algae, such as marine algae, which contain and express a desaturase transgene also are provided. For example, transgenic marine algae may be prepared as described in USPN 5,426,040. As with the other expression systems described above, the timing, extent of expression and activity of the desaturase transgene can be regulated by fitting the polypeptide coding sequence with the appropriate transcriptional and translational regulatory regions selected for a particular use. Of particular interest are promoter regions which can be induced under preselected growth conditions. For example, introduction of temperature sensitive and/or metabolite responsive mutations into the desaturase transgene coding sequences, its regulatory regions, and/or the genome of cells into which the transgene is introduced can be used for this purpose.

The transformed host cell is grown under appropriate conditions adapted for a desired end result. For host cells grown in culture, the conditions are typically optimized to produce the greatest or most economical yield of PUFAs, which relates to the selected desaturase activity. Media conditions which may be optimized include: carbon source, nitrogen source, addition of substrate, final concentration of added substrate, form of substrate added, aerobic or anaerobic growth, growth temperature, inducing agent, induction temperature, growth phase at induction, growth phase at harvest, pH, density, and maintenance of selection. Microorganisms of interest, such as yeast and algae are preferably grown in selected medium. For yeast, complex media such as peptone broth (YPD) or a defined media such as a minimal media (contains amino acids, yeast nitrogen base, and ammonium sulfate, and lacks a component for selection, for example uracil) are preferred. Desirably, substrates to be added are first dissolved in ethanol. Where necessary, expression of the polypeptide of interest may be induced, for example by including or adding galactose to induce expression from a GAL promoter.

### Expression In Animals And Animal Cells

Expression in cells of a host animal can likewise be accomplished in a transient or stable manner. Transient expression can be accomplished via known methods, for example infection or lipofection, and can be repeated in order to maintain desired expression levels of the introduced construct (*see* Ebert, PCT publication WO 94/05782). Stable expression can be accomplished via integration of a construct into the host genome, resulting in a transgenic animal. The construct can be introduced, for example, by microinjection of the construct into the pronuclei of a fertilized egg, or by transfection, retroviral infection or other techniques whereby the construct is introduced into a cell line which may form or be incorporated into an adult animal (U.S. Patent No. 4,873,191; U.S. Patent No. 5,530,177; U.S. Patent No. 5,565,362; U.S. Patent No. 5,366,894; Wilmut *et al.* (1997) *Nature* 385:810). The recombinant eggs or embryos are transferred to a surrogate mother (U.S. Patent No. 4,873,191; U.S. Patent No. 5,530,177; U.S. Patent No. 5,565,362; U.S. Patent No. 5,366,894; Wilmut *et al.* (supra)).

After birth, transgenic animals are identified, for example, by the presence of an introduced marker gene, such as for coat color, or by PCR or Southern blotting from a blood, milk or tissue sample to detect the introduced construct, or by an immunological or enzymological assay to detect the expressed protein or the products produced therefrom (U.S. Patent No. 4,873,191; U.S. Patent No. 5,530,177; U.S. Patent No. 5,565,362; U.S. Patent No. 5,366,894; Wilmut *et al.* (supra)). The resulting transgenic animals may be entirely transgenic or may be mosaics, having the transgenes in only a subset of their cells. The advent of mammalian cloning, accomplished by fusing a nucleated cell with an enucleated egg, followed by transfer into a surrogate mother, presents the possibility of rapid, large-scale production upon obtaining a "founder" animal or cell comprising the introduced construct; prior to this, it was necessary for the transgene to be present in the germ line of the animal for propagation (Wilmut *et al.* (supra)).

Expression in a host animal presents certain efficiencies, particularly where the host is a domesticated animal. For production of PUFAs in a fluid readily obtainable from the host animal, such as milk, the desaturase transgene can be expressed in mammary cells from a female host, and the PUFA content of the host cells altered. The desaturase transgene can be adapted for expression so that it is retained in the mammary cells, or secreted into milk, to form the PUFA reaction products localized to the milk (PCT publication WO 95/24488). Expression can be targeted for expression in mammary tissue using specific regulatory sequences, such as those of bovine  $\alpha$ -lactalbumin,  $\alpha$ -casein,  $\beta$ -casein,  $\gamma$ -casein,  $\kappa$ -casein,  $\beta$ -lactoglobulin, or whey acidic protein, and may optionally include one or more introns and/or secretory signal sequences (U.S. Patent No. 5,530,177; Rosen, U.S. Patent No. 5,565,362; Clark *et al.*, U.S. Patent No. 5,366,894; Garner *et al.*, PCT publication WO 95/23868). Expression of desaturase transgenes, or antisense desaturase transcripts, adapted in this manner can be used to alter the levels of specific PUFAs, or derivatives thereof, found in the animals milk. Additionally, the  $\Delta 5$ -desaturase transgene can be expressed either by itself or with other transgenes, in order to produce animal milk containing higher proportions of desired PUFAs or PUFA ratios and concentrations that resemble human breast milk (Prieto *et al.*, PCT publication WO 95/24494).

### PURIFICATION OF $\Delta 5$ DESATURASES

Recombinant  $\Delta 5$  desaturase may be purified from cell lysates and extracts, or from conditioned culture medium, by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography and hydrophobic interaction chromatography. Insect cell produced  $\Delta 5$  desaturase is a particularly good source of the purified enzyme. The purified  $\Delta 5$  desaturase may be used for the production of monoclonal and polyclonal antibodies.

In addition, recombinant  $\Delta 5$  desaturase can be separated from other cellular proteins by use of an immuno-affinity column made with monoclonal or

polyclonal antibodies specific for full length  $\Delta 5$  desaturase or polypeptide fragments of  $\Delta 5$  desaturase.

### **$\Delta 5$ DESATURASE ANTIBODIES**

Monospecific antibodies to  $\Delta 5$  desaturase are purified from mammalian antisera containing antibodies reactive against  $\Delta 5$  desaturase or are prepared as monoclonal antibodies reactive with  $\Delta 5$  desaturase, using procedures well known in the art such as the technique of Kohler and Milstein, Nature 256: 495-497 (1975). Monospecific antibody as used herein is defined as a single antibody species with homogenous binding characteristics for  $\Delta 5$  desaturase. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with the  $\Delta 5$  desaturase, as described above.  $\Delta 5$  desaturase specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with an appropriate concentration of  $\Delta 5$  desaturase either with or without an immune adjuvant.

### **Polyclonal Antibodies**

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1  $\mu\text{g}$  and about 1000  $\mu\text{g}$  of  $\Delta 5$  desaturase associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing *Corynebacterium parvum* and tRNA. The initial immunization consists of the  $\Delta 5$  desaturase protein in, preferably, Freund's complete adjuvant at multiple sites either subcutaneously (SC), intraperitoneally (IP) or both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of  $\Delta 5$  desaturase in Freund's incomplete adjuvant by the same route. Booster injections are given at about three week intervals until the maximal titers are obtained. At about 7 days after each booster immunization or about weekly

after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about -20° C.

### Monoclonal Antibodies

Monoclonal antibodies (mAb) reactive with  $\Delta 5$  desaturase are prepared  
5 by immunizing inbred mice, preferably Balb/c, with purified  $\Delta 5$  desaturase .  
The mice are immunized by the IP or SC route with about 1  $\mu$ g to about 100  $\mu$ g,  
preferably about 10  $\mu$ g, of  $\Delta 5$  desaturase in about 0.5 ml buffer or saline  
incorporated in an equal volume of an acceptable adjuvant, as discussed above.  
Freund's complete adjuvant is preferred. The mice receive an initial  
10 immunization on day 0 and are rested for about 3 to about 30 weeks.  
Immunized mice are given one or more booster immunizations of about 1 to  
about 100  $\mu$ g of  $\Delta 5$  desaturase in a buffer solution such as phosphate buffered  
saline by the intravenous (IV) route. Lymphocytes, from antibody positive  
mice, preferably splenic lymphocytes, are obtained by removing spleens from  
15 immunized mice by standard procedures known in the art. Hybridoma cells are  
produced by mixing the splenic lymphocytes with an appropriate fusion partner,  
preferably myeloma cells, under conditions which will allow the formation of  
stable hybridomas. Fusion partners may include, but are not limited to: mouse  
myelomas P3/NS1/Ag 4-1; MPC-11; S-104 and Sp 2/0, with Sp 2/0 being  
20 preferred.

The antibody producing cells and myeloma cells are fused in  
polyethylene glycol, about 1000 mol. wt., at concentrations from about 30% to  
about 50%. Fused hybridoma cells are selected by growth in hypoxanthine,  
thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium  
25 (DMEM) by procedures known in the art. Supernatant fluids are collect from  
growth positive wells on about days 14, 18 and 21 and are screened for  $\Delta 5$   
desaturase antibody production by an immunoassay such as solid phase  
immunoradioassay (SPIRA) using  $\Delta 5$  desaturase as the antigen. The culture  
fluids are also tested in the Ouchterlony precipitation assay to determine the  
30 isotype of the mAb. Hybridoma cells from antibody positive wells are cloned

by a technique such as the soft agar technique of MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, 1973.

Monoclonal antibodies are produced in vivo by injection of pristane  
5 primed Balb/c mice, approximately 0.5 ml per mouse, with about  $2 \times 10^6$  to about  $6 \times 10^6$  hybridoma cells about 4 days after priming. Ascites fluid is collected at approximately 8-12 days after cell transfer and the monoclonal antibodies are purified by techniques known in the art.

In vitro production of anti- $\Delta 5$  desaturase mAb is carried out by growing  
10 the hybridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited  
15 to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar assays are used to detect the presence of  $\Delta 5$  desaturases in body fluids or tissue and cell extracts.

It is readily apparent to those skilled in the art that the above described  
20 methods for producing monospecific antibodies may be utilized to produce antibodies specific for  $\Delta 5$  desaturase polypeptide fragments, or full-length  $\Delta 5$  desaturase polypeptide.

$\Delta 5$  desaturase antibody affinity columns are made by adding the antibodies to Affigel-10 (Biorad), a gel support which is pre-activated with N-  
25 hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23M glycine HCl (pH 2.6) to remove any non-conjugated  
30 antibody or extraneous protein. The column is then equilibrated in phosphate

buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing  $\Delta 5$  desaturase or  $\Delta 5$  desaturase fragments are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density (A280) falls to background, then the protein is eluted with  
5 0.23M glycine-HCl (pH 2.6). The purified  $\Delta 5$  desaturase protein is then dialyzed against phosphate buffered saline.

### PURIFICATION OF FATTY ACIDS

The fatty acids desaturated in the  $\Delta 5$  position may be found in the host microorganism or animal as free fatty acids or in conjugated forms such as  
10 acylglycerols, phospholipids, sulfolipids or glycolipids, and may be extracted from the host cell through a variety of means well-known in the art. Such means may include extraction with organic solvents, sonication, supercritical fluid extraction using for example carbon dioxide, and physical means such as presses, or combinations thereof. Of particular interest is extraction with  
15 methanol and chloroform. Where desirable, the aqueous layer can be acidified to protonate negatively charged moieties and thereby increase partitioning of desired products into the organic layer. After extraction, the organic solvents can be removed by evaporation under a stream of nitrogen. When isolated in conjugated forms, the products may be enzymatically or chemically cleaved to  
20 release the free fatty acid or a less complex conjugate of interest, and can then be subject to further manipulations to produce a desired end product. Desirably, conjugated forms of fatty acids are cleaved with potassium hydroxide.

If further purification is necessary, standard methods can be employed. Such methods may include extraction, treatment with urea, fractional  
25 crystallization, HPLC, fractional distillation, silica gel chromatography, high speed centrifugation or distillation, or combinations of these techniques. Protection of reactive groups, such as the acid or alkenyl groups, may be done at any step through known techniques, for example alkylation or iodination. Methods used include methylation of the fatty acids to produce methyl esters.  
30 Similarly, protecting groups may be removed at any step. Desirably,



purification of fractions containing ARA, DHA and EPA may be accomplished by treatment with urea and/or fractional distillation.

### USES OF FATTY ACIDS

There are several uses for fatty acids of the subject invention. Probes  
5 based on the DNAs of the present invention may find use in methods for  
isolating related molecules or in methods to detect organisms expressing  
desaturases. When used as probes, the DNAs or oligonucleotides must be  
detectable. This is usually accomplished by attaching a label either at an  
internal site, for example via incorporation of a modified residue, or at the 5' or  
10 3' terminus. Such labels can be directly detectable, can bind to a secondary  
molecule that is detectably labeled, or can bind to an unlabelled secondary  
molecule and a detectably labeled tertiary molecule; this process can be  
extended as long as is practical to achieve a satisfactorily detectable signal  
without unacceptable levels of background signal. Secondary, tertiary, or  
15 bridging systems can include use of antibodies directed against any other  
molecule, including labels or other antibodies, or can involve any molecules  
which bind to each other, for example a biotin-streptavidin/avidin system.  
Detectable labels typically include radioactive isotopes, molecules which  
chemically or enzymatically produce or alter light, enzymes which produce  
20 detectable reaction products, magnetic molecules, fluorescent molecules or  
molecules whose fluorescence or light-emitting characteristics change upon  
binding. Examples of labelling methods can be found in USPN 5,011,770.  
Alternatively, the binding of target molecules can be directly detected by  
measuring the change in heat of solution on binding of probe to target via  
25 isothermal titration calorimetry, or by coating the probe or target on a surface  
and detecting the change in scattering of light from the surface produced by  
binding of target or probe, respectively, as may be done with the BIAcore  
system.

PUFAs produced by recombinant means find applications in a wide  
30 variety of areas. Supplementation of humans or animals with PUFAs in various  
forms can result in increased levels not only of the added PUFAs, but of their

metabolic progeny as well. For example, where the inherent  $\Delta 5$ -desaturase pathway is dysfunctional in an individual, treatment with ARA can result not only in increased levels of ARA, but also of downstream products of ARA such as prostaglandins (see Figure 1). Complex regulatory mechanisms can make it desirable to combine various PUFAs, or to add different conjugates of PUFAs, in order to prevent, control or overcome such mechanisms to achieve the desired levels of specific PUFAs in an individual.

### FATTY ACID COMPOSITIONS

The present invention also includes fatty acid compositions such as nutritional compositions. Such compositions, for purposes of the present invention, include any food or preparation for human consumption including for enteral or parenteral consumption, which when taken into the body (a) serve to nourish or build up tissues or supply energy and/or (b) maintain, restore or support adequate nutritional status or metabolic function.

The nutritional composition of the present invention comprises at least one oil or acid produced in accordance with the present invention and may either be in a solid or liquid form. Additionally, the composition may include edible macronutrients, vitamins and minerals in amounts desired for a particular use. The amount of such ingredients will vary depending on whether the composition is intended for use with normal, healthy infants, children or adults having specialized needs such as those which accompany certain metabolic conditions (e.g., metabolic disorders).

Examples of macronutrients which may be added to the composition include but are not limited to edible fats, carbohydrates and proteins. Examples of such edible fats include but are not limited to coconut oil, soy oil, and mono- and diglycerides. Examples of such carbohydrates include but are not limited to glucose, edible lactose and hydrolyzed starch. Additionally, examples of proteins which may be utilized in the nutritional composition of the invention include but are not limited to soy proteins, electro dialysed whey, electro dialysed skim milk, milk whey, or the hydrolysates of these proteins.

With respect to vitamins and minerals, the following may be added to the nutritional compositions of the present invention: calcium, phosphorus, potassium, sodium, chloride, magnesium, manganese, iron, copper, zinc, selenium, iodine, and Vitamins A, E, D, C, and the B complex. Other such  
5 vitamins and minerals may also be added.

The components utilized in the nutritional compositions of the present invention will of semi-purified or purified origin. By semi-purified or purified is meant a material which has been prepared by purification of a natural material or by synthesis.

10 Examples of nutritional compositions of the present invention include but are not limited to infant formulas, dietary supplements, and rehydration compositions. Nutritional compositions of particular interest include but are not limited to those utilized for enteral and parenteral supplementation for infants, specialist infant formulae, supplements for the elderly, and supplements for  
15 those with gastrointestinal difficulties and/or malabsorption.

### **Nutritional Compositions**

A typical nutritional composition of the present invention will contain edible macronutrients, vitamins and minerals in amounts desired for a particular use. The amounts of such ingredients will vary depending on whether the  
20 formulation is intended for use with normal, healthy individuals temporarily exposed to stress, or to subjects having specialized needs due to certain chronic or acute disease states (e.g., metabolic disorders). It will be understood by persons skilled in the art that the components utilized in a nutritional formulation of the present invention are of semi-purified or purified origin. By  
25 semi-purified or purified is meant a material that has been prepared by purification of a natural material or by synthesis. These techniques are well known in the art (See, e.g., Code of Federal Regulations for Food Ingredients and Food Processing; Recommended Dietary Allowances, 10<sup>th</sup> Ed., National Academy Press, Washington, D.C., 1989).

In a preferred embodiment, a nutritional formulation of the present invention is an enteral nutritional product, more preferably an adult or child enteral nutritional product. Accordingly in a further aspect of the invention, a nutritional formulation is provided that is suitable for feeding adults, who are  
5 experiencing stress. The formula comprises, in addition to the PUFAs of the invention; macronutrients, vitamins and minerals in amounts designed to provide the daily nutritional requirements of adults.

The macronutritional components include edible fats, carbohydrates and proteins. Exemplary edible fats are coconut oil, soy oil, and mono- and  
10 diglycerides and the PUFA oils of this invention. Exemplary carbohydrates are glucose, edible lactose and hydrolyzed cornstarch. A typical protein source would be soy protein, electrodialysed whey or electrodialysed skim milk or milk whey, or the hydrolysates of these proteins, although other protein sources are also available and may be used. These macronutrients would be added in the  
15 form of commonly accepted nutritional compounds in amount equivalent to those present in human milk or an energy basis, i.e., on a per calorie basis.

Methods for formulating liquid and enteral nutritional formulas are well known in the art and are described in detail in the examples.

The enteral formula can be sterilized and subsequently utilized on a  
20 ready-to-feed (RTF) basis or stored in a concentrated liquid or a powder. The powder can be prepared by spray drying the enteral formula prepared as indicated above, and the formula can be reconstituted by rehydrating the concentrate. Adult and infant nutritional formulas are well known in the art and commercially available (e.g., Similac®, Ensure®, Jevity® and Alimentum®  
25 from Ross Products Division, Abbott Laboratories). An oil or acid of the present invention can be added to any of these formulas in the amounts described below.

The energy density of the nutritional composition when in liquid form, can typically range from about 0.6 Kcal to 3.0 Kcal per ml. When in solid or  
30 powdered form, the nutritional supplement can contain from about 1.2 to more than 9 Kcals per gm, preferably 3 to 7 Kcals per gram. In general, the

osmolality of a liquid product should be less than 700 mOsm and more preferably less than 660 mOsm.

The nutritional formula would typically include vitamins and minerals, in addition to the PUFAs of the invention, in order to help the individual ingest the minimum daily requirements for these substances. In addition to the PUFAs listed above, it may also be desirable to supplement the nutritional composition with zinc, copper, and folic acid in addition to antioxidants. It is believed that these substances will also provide a boost to the stressed immune system and thus will provide further benefits to the individual. The presence of zinc, copper or folic acid is optional and is not required in order to gain the beneficial effects on immune suppression. Likewise a pharmaceutical composition can be supplemented with these same substances as well.

In a more preferred embodiment, the nutritional contains, in addition to the antioxidant system and the PUFA component, a source of carbohydrate wherein at least 5 weight % of said carbohydrate is an indigestible oligosaccharide. In yet a more preferred embodiment, the nutritional composition additionally contains protein, taurine and carnitine.

The PUFAs, or derivatives thereof, made by the disclosed method can be used as dietary substitutes, or supplements, particularly infant formulas, for patients undergoing intravenous feeding or for preventing or treating malnutrition. Typically, human breast milk has a fatty acid profile comprising from about 0.15 % to about 0.36 % as DHA, from about 0.03 % to about 0.13 % as EPA, from about 0.30 % to about 0.88 % as ARA, from about 0.22 % to about 0.67 % as DGLA, and from about 0.27 % to about 1.04 % as GLA. Additionally, the predominant triglyceride in human milk has been reported to be 1,3-di-oleoyl-2-palmitoyl, with 2-palmitoyl glycerides reported as better absorbed than 2-oleoyl or 2-lineoyl glycerides (USPN 4,876,107). Thus, fatty acids such as ARA, DGLA, GLA and/or EPA produced by the invention can be used to alter the composition of infant formulas to better replicate the PUFA composition of human breast milk. In particular, an oil composition for use in a pharmacologic or food supplement, particularly a breast milk substitute or

supplement, will preferably comprise one or more of ARA, DGLA and GLA. More preferably the oil will comprise from about 0.3 to 30% ARA, from about 0.2 to 30% DGLA, and from about 0.2 to about 30% GLA.

In addition to the concentration, the ratios of ARA, DGLA and GLA can be adapted for a particular given end use. When formulated as a breast milk supplement, or substitute an oil composition which contains two or more of ARA, DGLA and GLA will be provided in a ratio of about 1:19:30 to about 6:1:0.2, respectively. For example, the breast milk of animals can vary in ratios of ARA:DGLA:DGL ranging from 1:19:30 to 6:1:0.2, which includes intermediate ratios which are preferably about 1:1:1, 1:2:1, 1:1:4. When produced together in a host cell, adjusting the rate and percent of conversion of a precursor substrate such as GLA and DGLA to ARA can be used to precisely control the PUFA ratios. For example, a 5% to 10% conversion rate of DGLA to ARA can be used to produce an ARA to DGLA ratio of about 1:19, whereas a conversion rate of about 75% to 80% can be used to produce an ARA to DGLA ratio of about 6:1. Therefore, whether in a cell culture system or in a host animal, regulating the timing, extent and specificity of desaturase expression as described can be used to modulate the PUFA levels and ratios. Depending on the expression system used, e.g., cell culture or an animal expressing oil(s) in its milk, the oils also can be isolated and recombined in the desired concentrations and ratios. Amounts of oils providing these ratios of PUFA can be determined following standard protocols. PUFAs, or host cells containing them, also can be used as animal food supplements to alter an animal's tissue or milk fatty acid composition to one more desirable for human or animal consumption.

For dietary supplementation, the purified PUFAs, or derivatives thereof, may be incorporated into cooking oils, fats or margarines formulated so that in normal use the recipient would receive the desired amount. The PUFAs may also be incorporated into infant formulas, nutritional supplements or other food products, and may find use as anti-inflammatory or cholesterol lowering agents.

### Pharmaceutical Compositions

The present invention also encompasses a pharmaceutical composition comprising one or more of the acids and/or resulting oils produced in accordance with the methods described herein. More specifically, such a  
5 pharmaceutical composition may comprise one or more of the acids and/or oils as well as a standard, well-known, non-toxic pharmaceutically acceptable carrier, adjuvant or vehicle such as, for example, phosphate buffered saline, water, ethanol, polyols, vegetable oils, a wetting agent or an emulsion such as a water/oil emulsion. The composition may be in either a liquid or solid form.  
10 For example, the composition may be in the form of a tablet, capsule, ingestible liquid or powder, injectible, or topical ointment or cream.

Possible routes of administration include, for example, oral, rectal and parenteral. The route of administration will, of course, depend upon the desired effect. For example, if the composition is being utilized to treat rough, dry, or  
15 aging skin, to treat injured or burned skin, or to treat skin or hair affected by a disease or condition, it may perhaps be applied topically.

The dosage of the composition to be administered to the patient may be determined by one of ordinary skill in the art and depends upon various factors such as weight of the patient, age of the patient, immune status of the patient,  
20 etc.

With respect to form, the composition may be, for example, a solution, a dispersion, a suspension, an emulsion or a sterile powder which is then reconstituted.

25

### Cosmetic Compositions

Additionally, the composition of the present invention may be utilized for cosmetic purposes. It may be added to pre-existing cosmetic compositions such that a mixture is formed or may be used as a sole composition.

The topical compositions into which PUFAs are formulated comprise a cosmetic or dermatological composition and can be provided in all conventional pharmaceutical dosage forms for topical application and the physiologically acceptable vehicle, diluent or carrier and therefore can be any standard vehicle or medium for a cosmetic or dermatological composition. The subject compositions can be formulated as an aqueous solution, or an oily suspension, or a dispersion of the lotion or serum type, or as an emulsion having a liquid or semi-liquid consistency of the milk type, obtained by dispersion of a fatty phase into an aqueous phase (O/W) or vice-versa (W/O), or as a suspension or emulsion having a soft consistency of the aqueous gel or cream type, or as microcapsules or microparticles, or as vesicular dispersions of ionic and/or non-ionic type. These compositions are formulated according to the usual techniques.

The amounts of the different constituents of the compositions are those conventionally employed in the fields under consideration.

The subject compositions constitute, in particular, cleansing, protection, treatment or care creams for the face, for the hands, for the major anatomical folds or for the body (for example day creams, night creams, makeup removal creams, foundation creams or anti-sun or sunscreen creams), fluid foundations, makeup removal milks, protection or care body milks, anti-sun or sunscreen milks, or lotions, gels or foams for caring for the skin, such as cleansing lotions, anti-sun lotions, artificial tanning lotions, and the like.

When the composition is an emulsion, the proportion of the fatty phase can range from 5% to 80% by weight and preferably from 5% to 50% by weight with respect to the total weight of the composition. The oils, waxes, emulsifiers and coemulsifiers formulated into the composition in the emulsion form are those conventional in the cosmetics field. The emulsifier and the coemulsifier are advantageously present in such compositions in a proportion ranging from 0.3% to 30% by weight and preferably from 0.5% to 20% by weight with respect to the total weight of the composition. The emulsion can, in addition, contain lipid vesicles.



The subject cosmetic or dermatological compositions can also contain adjuvants and additives usual in the cosmetics or dermatological field, such as hydrophilic or lipophilic gelling agents, hydrophilic or lipophilic additives, preservatives, antioxidants, solvents, fragrances, fillers, screening agents, odor absorbers and colorants. The amounts of these different adjuvants and additives are those conventional in these fields and, for example, range from 0.01% to 10% of the total weight of the composition. These adjuvants and additives, depending on their nature, can be introduced into the fatty phase, into the aqueous phase and/or into lipid spherules.

Exemplary oils and waxes include mineral oils (liquid petrolatum), vegetable oils (liquid fraction of karite butter, sunflower oil), animal oils (perhydrosqualene), synthetic oils (Purcellin oil), silicone oils or waxes (cyclomethicone) and fluorinated oils (perfluoropolyethers), beeswax or carnauba or parafin wax. Fatty alcohols and fatty acids (stearic acid) can be added to these oils.

Exemplary emulsifiers include, for example, glycerol stearate, polysorbate 60 and the PEG-6/PEG-31/glycol stearate mixture marketed under the trademark Tefose<sup>®</sup> 63 by Gattefosse.

Exemplary hydrophilic gelling agents according to the invention include the carboxyvinyl polymers (carbomer), acrylic copolymers, such as acrylate/alkyl acrylate copolymers, polyacrylamides, polysaccharides, such as hydroxypropylcellulose, natural gums and clays, and exemplary lipophilic gelling agents include the modified clays, such as bentones, or metal salts of fatty acids, such as aluminum stearates.

Insofar as they do not interfere or interact with the activity of the melatonin, the compositions of the present invention can contain other active ingredients suitable, in particular, for the prevention and/or for the treatment of skin conditions/afflictions.

The compositions according to the invention are particularly well suited for preventing or treating oxidative stress of the skin and/or its adnexa, in

aprticular related to UV irradiation, to aging, to inflammation, to alopecia, and the like.

### **Administration of the Pharmaceutical Compositions**

5           Pharmaceutical compositions may be utilized to administer the PUFA component to an individual. Suitable pharmaceutical compositions may comprise physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile solutions or dispersions for ingestion. Examples of suitable aqueous and  
10       non-aqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the maintenance of the required particle size in the case of dispersions and by the  
15       use of surfactants. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

              Suspensions, in addition to the active compounds, may contain  
20       suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth or mixtures of these substances, and the like.

              Solid dosage forms such as tablets and capsules can be prepared using  
25       techniques well known in the art. For example, PUFAs of the invention can be tableted with conventional tablet bases such as lactose, sucrose, and cornstarch in combination with binders such as acacia, cornstarch or gelatin, disintegrating agents such as potato starch or alginic acid and a lubricant such as stearic acid or magnesium stearate. Capsules can be prepared by incorporating these  
30       excipients into a gelatin capsule along with the antioxidants and the PUFA

component. The amount of the antioxidants and PUFA component that should be incorporated into the pharmaceutical formulation should fit within the guidelines discussed above.

As used in this application, the term "treat" refers to either preventing, or  
5 reducing the incidence of, the undesired occurrence. For example, to treat immune suppression refers to either preventing the occurrence of this suppression or reducing the amount of such suppression. The terms "patient" and "individual" are being used interchangeably and both refer to an animal. The term "animal" as used in this application refers to any warm-blooded  
10 mammal including, but not limited to, dogs, humans, monkeys, and apes. As used in the application the term "about" refers to an amount varying from the stated range or number by a reasonable amount depending upon the context of use. Any numerical number or range specified in the specification should be considered to be modified by the term about.

15 "Dose" and "serving" are used interchangeably and refer to the amount of the nutritional or pharmaceutical composition ingested by the patient in a single setting and designed to deliver effective amounts of the antioxidants and the structured triglyceride. As will be readily apparent to those skilled in the art, a single dose or serving of the liquid nutritional powder should supply the  
20 amount of antioxidants and PUFAs discussed above. The amount of the dose or serving should be a volume that a typical adult can consume in one sitting. This amount can vary widely depending upon the age, weight, sex or medical condition of the patient. However as a general guideline, a single serving or dose of a liquid nutritional produce should be considered as encompassing a  
25 volume from 100 to 600 ml, more preferably from 125 to 500 ml and most preferably from 125 to 300 ml.

### **Food Products**

The PUFAs of the present invention may also be added to food even when supplementation of the diet is not required. For example, the composition  
30 may be added to food of any type including but not limited to margarines,

modified butters, cheeses, milk, yogurt, chocolate, candy, snacks, salad oils, cooking oils, cooking fats, meats, fish and beverages.

### Pharmaceutical Applications

#### Administration

5           For pharmaceutical use (human or veterinary), the compositions are generally administered orally but can be administered by any route by which they may be successfully absorbed, e.g., parenterally (i.e. subcutaneously, intramuscularly or intravenously), rectally or vaginally or topically, for example, as a skin ointment or lotion. The PUFAs of the present invention may  
10 be administered alone or in combination with a pharmaceutically acceptable carrier or excipient. Where available, gelatin capsules are the preferred form of oral administration. Dietary supplementation as set forth above also can provide an oral route of administration. The unsaturated acids of the present invention may be administered in conjugated forms, or as salts, esters, amides  
15 or prodrugs of the fatty acids. Any pharmaceutically acceptable salt is encompassed by the present invention; especially preferred are the sodium, potassium or lithium salts. Also encompassed are the N-alkylpolyhydroxamine salts, such as N-methyl glucamine, found in PCT publication WO 96/33155. The preferred esters are the ethyl esters. As solid salts, the PUFAs also can be  
20 administered in tablet form. For intravenous administration, the PUFAs or derivatives thereof may be incorporated into commercial formulations such as Intralipids. The typical normal adult plasma fatty acid profile comprises 6.64 to 9.46% of ARA, 1.45 to 3.11% of DGLA, and 0.02 to 0.08% of GLA. These PUFAs or their metabolic precursors can be administered, either alone or in  
25 mixtures with other PUFAs, to achieve a normal fatty acid profile in a patient. Where desired, the individual components of formulations may be individually provided in kit form, for single or multiple use. A typical dosage of a particular fatty acid is from 0.1 mg to 20 g, or even 100 g daily, and is preferably from 10 mg to 1, 2, 5 or 10 g daily as required, or molar equivalent amounts of  
30 derivative forms thereof. Parenteral nutrition compositions comprising from about 2 to about 30 weight percent fatty acids calculated as triglycerides are

encompassed by the present invention; preferred is a composition having from about 1 to about 25 weight percent of the total PUFA composition as GLA (USPN 5,196,198). Other vitamins, and particularly fat-soluble vitamins such as vitamin A, D, E and L-carnitine can optionally be included. Where desired, a  
5 preservative such as  $\alpha$  tocopherol may be added, typically at about 0.1% by weight.

Suitable pharmaceutical compositions may comprise physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile injectible solutions  
10 or dispersions. Examples of suitable aqueous and non-aqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propyleneglyol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the maintenance of the required  
15 particle size in the case of dispersions and by the use of surfactants. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

20 Suspensions in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances and the like.

25 An especially preferred pharmaceutical composition contains diacetyltartaric acid esters of mono- and diglycerides dissolved in an aqueous medium or solvent. Diacetyltartaric acid esters of mono- and diglycerides have an HLB value of about 9-12 and are significantly more hydrophilic than existing antimicrobial lipids that have HLB values of 2-4. Those existing hydrophobic  
30 lipids cannot be formulated into aqueous compositions. As disclosed herein, those lipids can now be solubilized into aqueous media in combination with

diacetyltartaric acid esters of mono- and diglycerides. In accordance with this embodiment, diacetyltartaric acid esters of mono- and diglycerides (e.g., DATEM-C12:0) is melted with other active antimicrobial lipids (e.g., 18:2 and 12:0 monoglycerides) and mixed to obtain a homogeneous mixture.

- 5 Homogeneity allows for increased antimicrobial activity. The mixture can be completely dispersed in water. This is not possible without the addition of diacetyltartaric acid esters of mono- and diglycerides and premixing with other monoglycerides prior to introduction into water. The aqueous composition can then be admixed under sterile conditions with physiologically acceptable  
10 diluents, preservatives, buffers or propellants as may be required to form a spray or inhalant.

#### **Treatment of Diseases and Disorders**

- The present invention also encompasses the treatment of numerous disorders with fatty acids. Supplementation with PUFAs of the present  
15 invention can be used to treat restenosis after angioplasty. Symptoms of inflammation, rheumatoid arthritis, and asthma and psoriasis can be treated with the PUFAs of the present invention. Evidence indicates that PUFAs may be involved in calcium metabolism, suggesting that PUFAs of the present invention may be used in the treatment or prevention of osteoporosis and of  
20 kidney or urinary tract stones.

- The PUFAs of the present invention can be used in the treatment of cancer. Malignant cells have been shown to have altered fatty acid compositions; addition of fatty acids has been shown to slow their growth and cause cell death, and to increase their susceptibility to chemotherapeutic agents.  
25 GLA has been shown to cause reexpression on cancer cells of the E-cadherin cellular adhesion molecules, loss of which is associated with aggressive metastasis. Clinical testing of intravenous administration of the water soluble lithium salt of GLA to pancreatic cancer patients produced statistically significant increases in their survival. PUFA supplementation may also be  
30 useful for treating cachexia associated with cancer.

The PUFAs of the present invention can also be used to treat diabetes (USPN 4,826,877; Horrobin *et al.*, Am. J. Clin. Nutr. Vol. 57 (Suppl.), 732S-737S). Altered fatty acid metabolism and composition has been demonstrated in diabetic animals. These alterations have been suggested to be involved in some of the long-term complications resulting from diabetes, including retinopathy, neuropathy, nephropathy and reproductive system damage. Primrose oil, which contains GLA, has been shown to prevent and reverse diabetic nerve damage.

The PUFAs of the present invention can be used to treat eczema, reduce blood pressure and improve math scores. Essential fatty acid deficiency has been suggested as being involved in eczema, and studies have shown beneficial effects on eczema from treatment with GLA. GLA has also been shown to reduce increases in blood pressure associated with stress, and to improve performance on arithmetic tests. GLA and DGLA have been shown to inhibit platelet aggregation, cause vasodilation, lower cholesterol levels and inhibit proliferation of vessel wall smooth muscle and fibrous tissue (Brenner *et al.*, Adv. Exp. Med. Biol. Vol. 83, p. 85-101, 1976). Administration of GLA or DGLA, alone or in combination with EPA, has been shown to reduce or prevent gastro-intestinal bleeding and other side effects caused by non-steroidal anti-inflammatory drugs (USPN 4,666,701). GLA and DGLA have also been shown to prevent or treat endometriosis and premenstrual syndrome (USPN 4,758,592) and to treat myalgic encephalomyelitis and chronic fatigue after viral infections (USPN 5,116,871).

Further uses of the PUFAs of this invention include use in treatment of AIDS, multiple sclerosis, acute respiratory syndrome, hypertension and inflammatory skin disorders. The PUFAs of the inventions also can be used for formulas for general health as well as for geriatric treatments.

#### **Veterinary Applications**

It should be noted that the above-described pharmaceutical and nutritional compositions may be utilized in connection with animals, as well as humans, as animals experience many of the same needs and conditions as

human. For example, the oil or acids of the present invention may be utilized in animal feed supplements.

The following examples are presented by way of illustration, not of limitation.

5

### Examples

- |                    |   |
|--------------------|---|
| Example 1          | Isolation of a $\Delta 5$ -desaturase Nucleotide Sequence from <i>Mortierella alpina</i>                  |
| Example 2          | Expression of <i>M. alpina</i> $\Delta 5$ -desaturase Clones in Baker's Yeast                             |
| 10      Example 3  | Initial Optimization of Culture Conditions  |
| Example 4          | Distribution of PUFAs in Yeast Lipid Fractions  |
| Example 5          | Further Yeast Culture Optimization  |
| Example 6          | Expression of <i>M. alpina</i> $\Delta 5$ desaturase in insect cells                                      |
| 15      Example 7  | Identification of Homologues to <i>M. alpina</i> $\Delta 5$ and $\Delta 6$ desaturases                    |
| Example 8          | Identification of <i>M. alpina</i> $\Delta 5$ and $\Delta 6$ homologues in other PUFA-producing organisms |
| Example 9          | Identification of <i>M. alpina</i> $\Delta 5$ and $\Delta 6$ homologues in other PUFA-producing organisms |
| 20      Example 10 | Human Desaturase Sequences  |
| Example 11         | Production of Oil in Microorganisms   |
| Example 12         | Further Processing of Oil Produced by Microorganisms  |
| Example 13         | Further Purification of Oil Produced by Microorganisms  |
| Example 14         | Manufacture of Transgenic Oil Nutritional Products  |
| 25      Example 15 | Nutritional Compositions  |



### Example 1

#### Isolation of a $\Delta 5$ -desaturase Nucleotide Sequence from *Mortierella alpina*

*Mortierella alpina* produces arachidonic acid (ARA, 20:4) from the precursor 20:3 by a  $\Delta 5$ -desaturase. A nucleotide sequence encoding the  $\Delta 5$ -desaturase from *Mortierella alpina* was obtained through PCR amplification using *M. alpina* 1<sup>st</sup> strand cDNA and degenerate oligonucleotide primers corresponding to amino acid sequences conserved between  $\Delta 6$ -desaturases from *Synechocystis* and *Spirulina*. The procedure used was as follows:

Total RNA was isolated from a 3 day old PUFA-producing culture of *Mortierella alpina* using the protocol of Hoge *et al.* (1982) *Experimental Mycology* 6:225-232. The RNA was used to prepare double-stranded cDNA using BRL's lambda-ZipLox system, following the manufacturer's instructions. Several size fractions of the *M. alpina* cDNA were packaged separately to yield libraries with different average-sized inserts. The "full-length" library contains approximately  $3 \times 10^6$  clones with an average insert size of 1.77 kb. The "sequencing-grade" library contains approximately  $6 \times 10^5$  clones with an average insert size of 1.1 kb.

5  $\mu$ g of total RNA was reverse transcribed using BRL Superscript RTase and the primer TSyn (5'-CCAAGCTTCTGCAGGAGCTCTTTTTTTT TTTTTTTT-3'), SEQ ID NO:10. Degenerate oligonucleotides were designed to regions conserved between the two cyanobacterial  $\Delta 6$ -desaturase sequences. The specific primers used were D6DESAT-F3 (SEQ ID NO:8) (5'-CUACUACUACUACAYCAYACOTAYACOAAYAT-3') and D6DESAT-R3 (SEQ ID NO:9) (5'-CAUCAUCAUCAUOGGAAOARRTGRTG-3'), where Y=C+T, R=A+G, and O=I+C. PCR amplification was carried out in a 25  $\mu$ l volume containing: template derived from 40 ng total RNA, 2 pM each primer, 200  $\mu$ M each deoxyribonucleotide triphosphate, 60 mM Tris-Cl, pH 8.5, 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub>. Samples were subjected to an initial denaturation step of 95 degrees (all temperatures Celsius) for 5 minutes, then held at 72 degrees while 0.2 U of Taq polymerase were added. PCR thermocycling

conditions were as follows: 94 degrees for 1 min., 45 degrees for 1.5 min., 72 degrees for 2 min. PCR was continued for 35 cycles. PCR using these primers on the *M. alpina* first-strand cDNA produced a 550 bp reaction product.

Comparison of the deduced amino acid sequence of the *M. alpina* PCR

- 5 fragment SEQ ID NO:3 revealed regions of homology with  $\Delta 6$ -desaturases (see Figure 5). However, there was only about 28% identity over the region compared.

The PCR product was used as a probe to isolate corresponding cDNA clones from a *M. alpina* library. The longest cDNA clone, Ma29, was  
10 designated pCGN5521 and has been completely sequenced on both strands. The cDNA is contained as a 1481 bp insert in the vector pZL1 (Bethesda Research Laboratories) and, beginning with the first ATG, contains an open reading frame encoding 446 amino acids. The reading frame contains the sequence deduced from the PCR fragment. The sequence of the cDNA insert  
15 was found to contain regions of homology to  $\Delta 6$ -desaturases (see Figure 5). For example, three conserved "histidine boxes" (that have been observed in membrane-bound desaturases (Okuley *et al.*, (1994) *The Plant Cell* 6:147-158)) were found to be present in the *Mortierella* sequence at amino acid positions 171-175, 207-212, and 387-391 (see Figure 3). However, the typical  
20 "HXXHH" amino acid motif for the third histidine box for the *Mortierella* desaturase was found to be QXXHH, SEQ ID NO:11-12. Surprisingly, the amino-terminus of the encoded protein, showed significant homology to cytochrome b5 proteins. Thus, the *Mortierella* cDNA clone appears to represent a fusion between a cytochrome b5 and a fatty acid desaturase. Since  
25 cytochrome b5 is believed to function as the electron donor for membrane-bound desaturase enzymes, it is possible that the N-terminal cytochrome b5 domain of this desaturase protein is involved in its function. This may be advantageous when expressing the desaturase in heterologous systems for PUFA production.

## Example 2

### Expression of *M. alpina* Desaturase Clones in Baker's Yeast

#### Yeast Transformation

Lithium acetate transformation of yeast was performed according to standard protocols (*Methods in Enzymology*, Vol. 194, p. 186-187, 1991). Briefly, yeast were grown in YPD at 30°C. Cells were spun down, resuspended in TE, spun down again, resuspended in TE containing 100 mM lithium acetate, spun down again, and resuspended in TE/lithium acetate. The resuspended yeast were incubated at 30°C for 60 minutes with shaking. Carrier DNA was added, and the yeast were aliquoted into tubes. Transforming DNA was added, and the tubes were incubated for 30 min. at 30°C. PEG solution (35% (w/v) PEG 4000, 100 mM lithium acetate, TE pH7.5) was added followed by a 50 min. incubation at 30°C. A 5 min. heat shock at 42°C was performed, the cells were pelleted, washed with TE, pelleted again and resuspended in TE. The resuspended cells were then plated on selective media.

#### Desaturase Expression in Transformed Yeast

The cDNA clones from *Mortierella alpina* were screened for desaturase activity in baker's yeast. A canola  $\Delta 15$ -desaturase (obtained by PCR using 1<sup>st</sup> strand cDNA from *Brassica napus* cultivar 212/86 seeds using primers based on the published sequence (Arondel *et al. Science* 258:1353-1355)) was used as a positive control. The  $\Delta 15$ -desaturase gene and the gene from cDNA clone Ma29 was inserted into the expression vector pYES2 (Invitrogen), resulting in plasmids pCGR-2 and pCGR-4, respectively. These plasmids were transfected into *S. cerevisiae* yeast strain 334 and expressed after induction with galactose and in the presence of substrates that allowed detection of specific desaturase activity. The control strain was *S. cerevisiae* strain 334 containing the unaltered pYES2 vector. The substrates used, the products produced and the indicated desaturase activity were: DGLA (conversion to ARA would indicate  $\Delta 5$ -desaturase activity), linolenic acid (conversion to GLA would indicate  $\Delta 6$ -desaturase activity; conversion to ALA would indicate  $\Delta 15$ -desaturase activity),

oleic acid (an endogenous substrate made by *S. cerevisiae*, conversion to linolenic acid would indicate  $\Delta 12$ -desaturase activity, which *S. cerevisiae* lacks), or ARA (conversion to EPA would indicate  $\Delta 17$ -desaturase activity). The results are provided in Table 1 below.

5

### Lipid Extraction

The lipid fractions were extracted as follows: Cultures were grown for 48-52 hours at 15°C. Cells were pelleted by centrifugation, washed once with sterile ddH<sub>2</sub>O, and repelleted. Pellets were vortexed with methanol; chloroform was added along with tritridecanoin (as an internal standard). The mixtures were incubated for at least one hour at room temperature or at 4°C overnight. The chloroform layer was extracted and filtered through a Whatman filter with one gram of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated at 40°C under a stream of nitrogen. The extracted lipids were then derivatized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC) by adding 2 ml of 0.5 N potassium hydroxide in methanol to a closed tube. The samples were heated to 95°C to 100°C for 30 minutes and cooled to room temperature. Approximately 2 ml of 14 % boron trifluoride in methanol was added and the heating repeated. After the extracted lipid mixture cooled, 2 ml of water and 1 ml of hexane were added to extract the FAME for analysis by GC. The percent conversion was calculated by dividing the product produced by the sum of (the product produced and the substrate added) and then multiplying by 100. To calculate the oleic acid percent conversion, as no substrate was added, the total linolenic acid produced was divided by the sum of (oleic acid and linolenic acid produced), then multiplying by 100.

**Table 1*****M. alpina* Desaturase Expression in Baker's Yeast**

| CLONE                              | TYPE OF ENZYME<br>ACTIVITY | % CONVERSION<br>OF SUBSTRATE    |
|------------------------------------|----------------------------|---------------------------------|
| pCGR-2                             | $\Delta 6$                 | 0 (18:2 to 18:3 $\omega 6$ )    |
| (canola $\Delta 15$<br>desaturase) | $\Delta 15$                | 16.3 (18:2 to 18:3 $\omega 3$ ) |
|                                    | $\Delta 5$                 | 2.0 (20:3 to 20:4 $\omega 6$ )  |
|                                    | $\Delta 17$                | 2.8 (20:4 to 20:5 $\omega 3$ )  |
|                                    | $\Delta 12$                | 1.8 (18:1 to 18:2 $\omega 6$ )  |
| pCGR-4                             | $\Delta 6$                 | 0                               |
| (M. alpina<br>Ma29)                | $\Delta 15$                | 0                               |
|                                    | $\Delta 5$                 | 15.3                            |
|                                    | $\Delta 17$                | 0.3                             |
|                                    | $\Delta 12$                | 3.3                             |

The  $\Delta 15$ -desaturase control clone exhibited 16.3% conversion of the  
 5 substrate. The pCGR-4 clone expressing the Ma29 cDNA converted 15.3% of  
 the 20:3 substrate to 20:4 $\omega 6$ , indicating that the gene encodes a  $\Delta 5$ -desaturase.  
 The background (non-specific conversion of substrate) was between 0-3% in  
 these cases. We also found substrate inhibition of the activity by using  
 different concentrations of the substrate. When substrate was added to 100  $\mu\text{M}$ ,  
 10 the percent conversion to product dropped compared to when substrate was  
 added to 25  $\mu\text{M}$  (see below). Additionally, by varying the DGLA substrate  
 concentrations, between about 5  $\mu\text{M}$  to about 200  $\mu\text{M}$  percent conversion of  
 DGLA to ARA ranged from about 5% to 75% with the *M. alpina*  $\Delta 5$ -  
 desaturase.

15 These data show that desaturases with different substrate specificities  
 can be expressed in a heterologous system and used to produce poly-unsaturated  
 long chain fatty acids.

Table 2 represents fatty acids of interest as a percent of the total lipid extracted from the yeast host *S. cerevisiae* 334 with the indicated plasmid. No glucose was present in the growth media. Affinity gas chromatography was used to separate the respective lipids. GC/MS was employed to verify the identity product(s). The expected product for the *B. napus*  $\Delta 15$ -desaturase,  $\alpha$ -linolenic acid, was detected when its substrate, linolenic acid, was added exogenously to the induced yeast culture. This finding demonstrates that yeast expression of a desaturase gene can produce functional enzyme and detectable amounts of product under the current growth conditions. Both exogenously added substrates were taken up by yeast, although slightly less of the longer chain PUFA, dihomo- $\gamma$ -linolenic acid (20:3), was incorporated into yeast than linolenic acid (18:2) when either was added in free form to the induced yeast cultures. Arachidonic acid was detected as a novel PUFA in yeast when dihomo- $\gamma$ -linolenic acid was added as the substrate to *S. cerevisiae* 334 (pCGR-4). This identifies pCGR-4 (MA29) as the  $\Delta 5$ -desaturase from *M. alpina*. Prior to this, no isolation and expression of a  $\Delta 5$ -desaturase from any source has been reported.

Table 2  
Fatty Acid as a Percentage of Total Lipid Extracted from Yeast

| Plasmid<br>in Yeast<br>(enzyme) | 18:2<br>Incorporated | $\alpha$ -18:3<br>Produced | $\gamma$ -18:3<br>Produced | 20:3<br>Incorporated | 20:4<br>Produced | 18:1*<br>Present | 18:2<br>Produced |
|---------------------------------|----------------------|----------------------------|----------------------------|----------------------|------------------|------------------|------------------|
| pYES2<br>(control)              | 66.9                 | 0                          | 0                          | 58.4                 | 0                | 4                | 0                |
| pCGR-2<br>( $\Delta 15$ )       | 60.1                 | 5.7                        | 0                          | 50.4                 | 0                | 0.7              | 0                |
| pCGR-4<br>( $\Delta 5$ )        | 67                   | 0                          | 0                          | 32.3                 | 5.8              | 0.8              | 0                |

100  $\mu$ M substrate added

\* 18:1 is an endogenous fatty acid in yeast

#### Key To Tables

18:1 =oleic acid  
 18:2 =linolenic acid  
 $\alpha$ -18:3 = $\alpha$ -linolenic acid  
 $\gamma$ -18:3 = $\gamma$ -linolenic acid  
 18:4 =stearidonic acid  
 20:3 =dihomo- $\gamma$ -linolenic acid  
 20:4 =arachidonic acid

**Example 3****Optimization of Culture Conditions**

Table 3A shows the effect of exogenous free fatty acid substrate concentration on yeast uptake and conversion to fatty acid product as a percentage of the total yeast lipid extracted. In all instances, low amounts of exogenous substrate (1-10  $\mu$ M) resulted in low fatty acid substrate uptake and product formation. Between 25 and 50  $\mu$ M concentration of free fatty acid in the growth and induction media gave the highest percentage of fatty acid product formed, while the 100  $\mu$ M concentration and subsequent high uptake into yeast appeared to decrease or inhibit the desaturase activity. The feedback inhibition of high fatty acid substrate concentration was well illustrated when the percent conversion rates of the respective fatty acid substrates to their respective products were compared in Table 3B. In all cases, 100  $\mu$ M substrate concentration in the growth media decreased the percent conversion to product. The effect of media composition was also evident when glucose was present in the growth media for the  $\Delta 5$ -desaturase, since the percent of substrate uptake was decreased at 25  $\mu$ M (Table 3A). However, the percent conversion by  $\Delta 5$ -desaturase increased by 18% and the percent product formed remained the same in the presence of glucose in the growth media.



**Table 3A**  
**Effect of Added Substrate on the Percentage of Incorporated**  
**Substrate and Product Formed in Yeast Extracts**

| Plasmid in Yeast           | pCGR-2<br>( $\Delta 15$ ) | pCGR-4<br>( $\Delta 5$ ) |
|----------------------------|---------------------------|--------------------------|
| substrate/product          | 18:2 / $\alpha$ -18:3     | 20:3/20:4                |
| 1 $\mu$ M sub.             | ND                        | 0.5/1.7                  |
| 10 $\mu$ M sub.            | ND                        | 3.3/4                    |
| 25 $\mu$ M sub.            | ND                        | 5.1/6.1                  |
| 25 $\mu$ M $\diamond$ sub. | 36.6/7.2 $\diamond$       | 9.3/5.4 $\diamond$       |
| 50 $\mu$ M sub.            | 53.1/6.5 $\diamond$       | ND                       |
| 100 $\mu$ M sub.           | 60.1/5.7 $\diamond$       | 32.3/5.8 $\diamond$      |

5

**Table 3B**  
**Effect of Substrate Concentration in Media on the Percent Conversion**  
**of Fatty Acid Substrate to Product in Yeast Extracts**

| Plasmid in Yeast           | pCGR-2<br>( $\Delta 15$ )         | pCGR-4<br>( $\Delta 5$ ) |
|----------------------------|-----------------------------------|--------------------------|
| substrate/product          | 18:2 $\rightarrow$ $\alpha$ -18:3 | 20:3 $\rightarrow$ 20:4  |
| 1 $\mu$ M sub.             | ND                                | 77.3                     |
| 10 $\mu$ M sub.            | ND                                | 54.8                     |
| 25 $\mu$ M sub.            | ND                                | 54.2                     |
| 25 $\mu$ M $\diamond$ sub. | 16.4                              | 36.7                     |
| 50 $\mu$ M sub.            | 10.9 $\diamond$                   | ND                       |
| 100 $\mu$ M sub.           | 8.7 $\diamond$                    | 15.2 $\diamond$          |

$\diamond$  no glucose in media

\* Yeast peptone broth (YPD)

\* 18:1 is an endogenous yeast lipid

sub. is substrate concentration

ND (not done)

10

15 Table 4 shows the amount of fatty acid produced by a recombinant desaturase from induced yeast cultures when different amounts of free fatty acid substrate were used. Fatty acid weight was determined since the total amount of lipid varied dramatically when the growth conditions were changed, such as the presence of glucose in the yeast growth and induction media. To better determine

the conditions when the recombinant desaturase would produce the most PUFA product, the quantity of individual fatty acids were examined. The absence of glucose reduced the amount of arachidonic acid produced by  $\Delta 5$ -desaturase by half. For  $\Delta 5$ -desaturase the amount of total yeast lipid was decreased by almost half in the absence of glucose.

Table 4

**Fatty Acid Produced in  $\mu\text{g}$  from Yeast Extracts**

| Plasmid in Yeast<br>(enzyme)     | pCGR-4<br>( $\Delta 5$ ) | pCGR-7<br>( $\Delta 12$ ) |
|----------------------------------|--------------------------|---------------------------|
| Product                          | 20:4                     | 18:2*                     |
| 1 $\mu\text{M}$ sub.             | 8.3                      | ND                        |
| 10 $\mu\text{M}$ sub.            | 19.2                     | ND                        |
| 25 $\mu\text{M}$ sub.            | 31.2                     | 115.7                     |
| 25 $\mu\text{M}$ $\diamond$ sub. | 16.8                     | 39 $\diamond$             |

$\diamond$  no glucose in media

sub. is substrate concentration

ND (not done)

\*18:1, the substrate, is an endogenous yeast lipid

**Example 4****Distribution of PUFAs in Yeast Lipid Fractions**

Table 5 illustrates the uptake of free fatty acids and their new products formed in yeast lipids as distributed in the major lipid fractions. A total lipid extract was prepared as described above. The lipid extract was separated on TLC plates, and the fractions were identified by comparison to standards. The bands were collected by scraping, and internal standards were added. The fractions were then saponified and methylated as above, and subjected to gas chromatography. The gas chromatograph calculated the amount of fatty acid by comparison to a standard. It would appear that the substrates are accessible in the phospholipid form to the desaturases.

**Table 5**  
**Fatty Acid Distribution in Various Yeast Lipid Fractions in  $\mu\text{g}$**

| Fatty acid fraction        | Phospholipid | Diglyceride | Free Fatty Acid | Triglyceride | Cholesterol Ester |
|----------------------------|--------------|-------------|-----------------|--------------|-------------------|
| SC (pCGR-4) substrate 20:3 | 15.1         | 1.9         | 22.9            | 12.6         | 3.3               |
| SC (pCGR-4) product 20:4   | 42.6         | 0.9         | 6.8             | 4.9          | 0.4               |

SC = *S. cerevisiae* (plasmid)

5

### Example 5

#### Further Yeast Culture Optimization

The growth and induction conditions for optimal activities of desaturases in *Saccharomyces cerevisiae* were evaluated. Various culture conditions that were manipulated for optimal activity were: I) induction temperature, ii) concentration of inducer, iii) timing of substrate addition, iv) concentration of substance, v) sugar source, vi) growth phase at induction. These studies were done using  $\Delta 5$ -desaturase gene from *Mortierella alpina* (MA 29). In addition, the effect of changing host strain on expression of the  $\Delta 5$ -desaturase gene was also determined.

As described above, the best rate of conversion of substrate to ARA was observed at a substrate concentration of 1  $\mu\text{M}$ , however, the percentage of ARA in the total fatty acids was highest at 25  $\mu\text{M}$  substrate concentration. To determine if the substrate needed to be modified to a readily available form before it could be utilized by the desaturase, the substrate was added either 15 hours before induction or concomitant with inducer addition (indicated as after, in Figure 6A). As it can be seen in Figure 6A, addition of substrate before induction did not have a significant effect on the activity of  $\Delta 5$ -desaturase. In fact, addition of substrate along with the inducer was slightly better for expression/activity of  $\Delta 5$ -desaturase, as ARA levels in the total fatty acids were higher. However, the rate of conversion of substrate to product was slightly lower.

The effect of inducer concentration of expression/activity of *Mortierella*  $\Delta 5$ -desaturase was examined by inducing SC334/pCGR5 with 0.5 or 2% (w/v) of galactose. As shown in Figures 7A and 7B, expression of  $\Delta 5$ -desaturase was higher when induced with 0.5% galactose. Furthermore, rate of conversion of substrate to product was also better when SC334/pCGR5 was induced with 0.5% galactose vs 2% galactose.

To determine the effect of temperature on  $\Delta 5$ -desaturase activity, the SC334 host strain, transformed with pCGR5 (SC334/pCGR5) was grown and induced at 15° C, 25°C, 30°C and 37°C. The quantity of ARA (20:4n6) produced in SC334/pCGR5 cultures, supplemental with substrate 20:3n6, was measured by fatty acid analysis. Figure 8A depicts the quantity of 20:3n6 and 20:4n6, expressed as percentage of total fatty acids. Figure 8B depicts the rate of conversion of substrate to product. Growth and induction of SC334/pCGR5 at 25°C, was the best for the expression of  $\Delta 5$ -desaturase as evidenced by the highest levels of arachidonic acid in the total fatty acids. Additionally the highest rate of conversion of substrate to product also occurred at 25°C. Growth and induction at 15°C gave the lowest expression of ARA, whereas at 37°C gave the lowest conversion of substrate to product.

The effect of yeast strain on expression of the  $\Delta 5$ -desaturase gene was studied in 5 different host strains; INVSC1, INVSC2, YTC34, YTC41, and SC334, at 15°C and 30°C. At 15°C, SC334 has the highest percentage of ARA in total fatty acids, suggesting higher activity of  $\Delta 5$ -desaturase in SC334. The rate of conversion of substrate to product, however is lowest in SC334 and highest in INVSC1 (Fig. 9A and B). At 30°C, the highest percentage of product (ARA) in total fatty acids was observed in INVSC2, although the rate of conversion of substrate to product in INVSC2 was slightly lower than INVSC1 (Fig. 10A and B).

ARA, the product of  $\Delta 5$ -desaturase, is stored in the phospholipid fraction (Example 4). Therefore the quantity of ARA produced in yeast is limited by the amount that can be stored in the phospholipid fraction. If ARA could also be stored in other fractions such as the triglyceride fraction, the quantity of ARA produced in yeast might be increased. To test this hypothesis, the  $\Delta 5$ -desaturase

gene was expressed in the yeast host strain DBY746 (obtained from the Yeast Genetic Stock Centre, 1021 Donner Laboratory, Berkeley, CA 94720. The genotype of strain DBY746 is Mat $\alpha$ , his3- $\Delta$ 1, leu2-3, leu2-112, ura3-32, trp1-289, gal). The DBY746 yeast strain has an endogenous gene for choline transferase.

5 The presence of this enzyme might enable the DBY746 strain to convert excess phospholipids into triglycerides fraction. Results in Fig. 11 show no increase in the conversion of substrate to product as compared to SC334, which does not have the gene for choline transferase.

To study the effect of media on expression of  $\Delta$ 5-desaturase,

10 pCGR4/SC334 was grown in four different media at two different temperatures (15°C and 30°) and in two different host strains (SC334 and INVSC1). The composition of the media was as follows:

Media A: mm-Ura, + 2% galactose + 2% glucose.

Media B: mm-Ura, + 20% galactose + 2% Glucose + 1M sorbitol (pH5.8)

15 Media C: mm-Ura, + 2% galactose + 2% raffinose

Media D: mm-Ura, + 2% galactose + 2% raffinose + 1M sorbitol (pH5.8)

mm=minimal media

Results show that the highest conversion rate of substrate to product at 15°C in SC334 was observed in media A. The highest conversion rate overall for

20  $\Delta$ 5-desaturase in SC334 was at 30° in media D. The highest conversion rate of  $\Delta$ 5-desaturase in INVSC1 was also at 30° in media D (Figures 12A and 12B).

These data show that a DNA encoding a desaturase that can convert DGLA to ARA can be isolated from *Mortierella alpina* and can be expressed in a heterologous system and used to produce poly-unsaturated long chain fatty acids.

25 Exemplified is the production of ARA from the precursor DGLA by expression of a  $\Delta$ 5-desaturase in yeast.

### Example 6

#### Expression Of *M. alpina* $\Delta 5$ Desaturases In Insect Cells

Insect cells were used as another eukaryotic host for expression of the *M. alpina* D5-desaturase. The baculovirus expression system involves the use of insect cells to express a gene, in this case the *M. alpina* D5-desaturase, which has been cloned into a baculovirus expression vector. Insect cells are known to have no endogenous PUFA desaturase activities. Therefore, this system is suitable for expression and characterization of the recombinant desaturases.

The fragment containing the D5-desaturase gene (pCGR4, see Example 2) was PCR amplified using Expand High Fidelity PCR System (Boehringer Mannheim, Corp., Indianapolis, IN) and a set of primers containing appropriate restriction sites. The upstream primer designated RO651 (5'-ATACCGGAATTCGCCGCCACCATGGGAACGGACCAAGGAAAAAC), SEQ ID NO: 35 corresponded to the sense strand of D5 cDNA and contained an EcoRI site 5' upstream of the ATG. The downstream primer RO652 (5'-TATCCGCTCGAGCTACTCTTCCTTGGGACGGAG), SEQ ID NO: 36 corresponded to the antisense strand at the 3' end of the D5 cDNA, and included an XhoI site immediately downstream of the translational termination codon. The PCR reaction, in a final volume of 100  $\mu$ l, was carried out for 30 cycles in temperature conditions of 45 seconds at 94°C, 45 seconds at 55°C and 2 min at 72°C. The D5 PCR amplified product was analyzed by agarose-gel electrophoresis, gel purified, digested with EcoRI and XhoI, and then ligated into pFastBac1 baculovirus donor plasmid (Gibco-BRL, Gaithersburg, MD) which was restricted with the same enzymes. The respective baculovirus clone was designated as pJPB5 for the D5-desaturase. This pFastBac1 vector contains an expression cassette which has a polyhedrin promoter, a SV40 polyadenylation signal, and a gentamycin resistance marker.

The initial transformation was done in XL1 blue cells (Invitrogen, Carlsbad, CA). Positive clones were then transformed into *E. coli* DH10Bac (Gibco-BRL, Gaithersburg, MD) which contains the baculovirus genome. The positive clones were selected by blue white screening in which white colonies

contain the recombinant bacmid. White colonies were then selected for bacmid DNA isolation. DNA was isolated using a Qiagen plasmid isolation kit (Qiagen Inc., Valencia, CA), specific for DNA over 135 kb long. The recombinant DNA bacmid was analyzed on a 0.6% agarose gel to confirm the presence of the high molecular weight DNA. PCR analysis, using pUC/M13 primers (forward 5'-TGTAACGACGGCCAGT SEQ ID NO: 37 and reverse 5'-GAAACAGCTATGACCATG) SEQ ID NO: 38 was also performed to confirm the correct insert size for the desaturase cDNA within the bacmid.

The Sf9 insect cells (*Spodoptera frugiperda*) were used for the recombinant bacmid DNA transfection. These cells were grown in serum free media Sf900 (Gibco BRL, Gaithersburg, MD). Transfection was carried out according to the CellFECTIN protocol (Gibco-BRL, Gaithersburg, MD). The recombinant virus was recovered by collecting the supernatant at 72 hours post-transfection. A Plaque assay was performed on the supernatant to determine the titer of recovered recombinant virion particles. A recombinant viral stock was made for the expression studies. All infections with the recombinant virus were done during the mid-logarithmic growth phase of the Sf9's and infected at 5 MOI (Multiplicity of Infection). To analyze the activity of the expressed D5-desaturase gene, the Sf9 cells were plated at a concentration of  $1 \times 10^6$  cells/well in a 6-well tissue culture plate and infected with 100ul of the virus stock (approximately 5 MOI). The substrate, dihomogamma-linolenic acid (DGLA, C20:3n-6) was supplemented at the time of infection, at a concentration of 25  $\mu$ M. A mock infected Sf9, as well as cells infected with a recombinant virus containing the GusA reporter gene, were used as negative controls in each experiment. The medium was collected 48 hours post infection and saved. The cells were collected and submitted for lipid analysis.

For fatty acid analysis, cell pellets were vortexed with 6 ml of methanol, followed by the addition of 12 ml of chloroform and tridecanoin (as internal standard). The mixtures were incubated for at least one hour at room temperature or at 4°C overnight. The chloroform layer was extracted and filtered through a Whatman filter with one gram of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated at 400°C under a stream of nitrogen. The extracted lipids were derivatized to fatty acid methyl esters

(FAME) for gas chromatography analysis (GC) by adding 2 ml of 0.5N potassium hydroxide in methanol to a closed tube. The samples were heated at 95 to 100°C for 30 minutes and cooled to room temperature. Approximately 2 ml of the 14% boron trifluoride in methanol was added and the heating repeated. After the extracted lipid mixture cooled, 2 ml of water and 1 ml of hexane were added to extract the FAME for GC analysis. The percent conversion was calculated by dividing the product produced by the sum of (the product produced and the substrate) and then multiplying by 100.

The fatty acid synthesis in insect cells infected with recombinant virus containing D5 cDNA is summarized in Table 6. The conversion of the added substrate, DGLA, to Arachidonic acid (AA, 20:4n-6) was monitored. AA, and other fatty acids containing longer carbon chains, were detected in the presence of the recombinant fungal D5-desaturase enzyme. The quantity of arachidonic acid (AA, 20:4n-6) produced by the D5-desaturase was 1.4% of the total fatty acid versus the control which expressed only 0.175% . This resulted in a 23% conversion of DGLA to AA.

These data indicate that the *M. alpina* D5-desaturase can be expressed in another eukaryotic host (insect cells) in a biologically active form as demonstrated by the production of AA as well as the accumulation of fatty acids with longer carbon chains.

**Table 6**

| Fatty Acid | $\Delta 5$ | Control |
|------------|------------|---------|
| *20:3n-6   | 27.4       | 26.3    |
| 20:4n-6    | (23%) 1.40 | 0.175   |
| 20:4n-3    | 0.090      | 0.08    |
| 20:5n-3    | 0.200      | 0.13    |
| 22:5n-3    | 0.027      | 0.015   |



### Example 7

#### Identification of Homologues to *M. alpina* $\Delta 5$ and $\Delta 6$ desaturases

A nucleic acid sequence that encodes a putative  $\Delta 5$  desaturase was identified through a TBLASTN search of the est databases through NCBI using amino acids 100-446 of Ma29 as a query. The truncated portion of the Ma29 sequence was used to avoid picking up homologies based on the cytochrome b5 portion at the N-terminus of the desaturase. The deduced amino acid sequence of an est from *Dictyostelium discoideum* (accession # C25549) shows very significant homology to Ma29 and lesser, but still significant homology to Ma524. The DNA sequence is presented as SEQ ID NO:13. The amino acid sequence is presented as SEQ ID NO:14.

### Example 8

#### Identification of *M. alpina* $\Delta 5$ and $\Delta 6$ homologues in other PUFA-producing organisms

To look for desaturases involved in PUFA production, a cDNA library was constructed from total RNA isolated from *Phaeodactylum tricornutum*. A plasmid-based cDNA library was constructed in pSPORT1 (GIBCO-BRL) following manufacturer's instructions using a commercially available kit (GIBCO-BRL). Random cDNA clones were sequenced and nucleic acid sequences that encode putative  $\Delta 5$  or  $\Delta 6$  desaturases were identified through BLAST search of the databases and comparison to Ma29 and Ma524 sequences.

One clone was identified from the *Phaeodactylum* library with homology to Ma29 and Ma524; it is called 144-011-B12. The DNA sequence is presented as SEQ ID NO:15. The amino acid sequence is presented as SEQ ID NO:16.

### Example 9

#### Identification of *M. alpina* $\Delta 5$ and $\Delta 6$ homologues in other PUFA-producing organisms

To look for desaturases involved in PUFA production, a cDNA library was  
5 constructed from total RNA isolated from *Schizochytrium* species. A  
plasmid-based cDNA library was constructed in pSPORT1 (GIBCO-BRL)  
following manufacturer's instructions using a commercially available kit  
(GIBCO-BRL). Random cDNA clones were sequenced and nucleic acid  
sequences that encode putative  $\Delta 5$  or  $\Delta 6$  desaturases were identified through  
10 BLAST search of the databases and comparison to Ma29 and Ma524 sequences.

One clone was identified from the *Schizochytrium* library with homology to  
Ma29 and Ma524; it is called 81-23-C7. This clone contains a ~1 kb insert. Partial  
sequence was obtained from each end of the clone using the universal forward and  
reverse sequencing primers. The DNA sequence from the forward primer is  
15 presented as SEQ ID NO:17. The peptide sequence is presented as SEQ ID  
NO:18. The DNA sequence from the reverse primer is presented as SEQ ID  
NO:19. The amino acid sequence from the reverse primer is presented as SEQ ID  
NO:20.

### Example 10

#### Human Desaturase Gene Sequences

Human desaturase gene sequences potentially involved in long chain  
polyunsaturated fatty acid biosynthesis were isolated based on homology between  
the human cDNA sequences and *Mortierella alpina* desaturase gene sequences.  
The three conserved "histidine boxes" known to be conserved among membrane-  
25 bound desaturases were found. As with some other membrane-bound desaturases  
the final HXXHH histidine box motif was found to be QXXHH. The amino acid  
sequence of the putative human desaturases exhibited homology to *M. alpina*  $\Delta 5$ ,  
 $\Delta 6$ ,  $\Delta 9$ , and  $\Delta 12$  desaturases.

The *M. alpina*  $\Delta 5$  desaturase and  $\Delta 6$  desaturase cDNA sequences were used to search the LifeSeq database of Incyte Pharmaceuticals, Inc., Palo Alto, California 94304. The  $\Delta 5$  desaturase sequence was divided into fragments; 1) amino acid no. 1-150, 2) amino acid no. 151-300, and 3) amino acid no. 301-446. The  $\Delta 6$  desaturase sequence was divided into three fragments; 1) amino acid no. 1-150, 2) amino acid no. 151-300, and 3) amino acid no. 301-457. These polypeptide fragments were searched against the database using the "tblastn" algorithm. This algorithm compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).

The polypeptide fragments 2 and 3 of *M. alpina*  $\Delta 5$  and  $\Delta 6$  have homologies with the CloneID sequences as outlined in Table 6. The CloneID represents an individual sequence from the Incyte LifeSeq database. After the "tblastn" results have been reviewed, Clone Information was searched with the default settings of Stringency of  $\geq 50$ , and Productscore  $\leq 100$  for different CloneID numbers. The Clone Information Results displayed the information including the ClusterID, CloneID, Library, HitID, Hit Description. When selected, the ClusterID number displayed the clone information of all the clones that belong in that ClusterID. The Assemble command assembles all of the CloneID which comprise the ClusterID. The following default settings were used for GCG (Genetics Computer Group, University of Wisconsin Biotechnology Center, Madison, Wisconsin 53705) Assembly:

|                   |     |
|-------------------|-----|
| Word Size:        | 7   |
| Minimum Overlap:  | 14  |
| Stringency:       | 0.8 |
| Minimum Identity: | 14  |
| Maximum Gap:      | 10  |
| Gap Weight:       | 8   |
| Length Weight:    | 2   |

GCG Assembly Results displayed the contigs generated on the basis of sequence information within the CloneID. A contig is an alignment of DNA sequences based on areas of homology among these sequences. A new sequence (consensus sequence) was generated based on the aligned DNA sequences within a contig. The contig containing the CloneID was identified, and the ambiguous sites of the consensus sequence was edited based on the alignment of the CloneIDs (see SEQ ID NO:21 - SEQ ID NO:25) to generate the best possible sequence. The procedure was repeated for all six CloneID listed in Table 6. This produced five unique contigs. The edited consensus sequences of the 5 contigs were imported into the Sequencher software program (Gene Codes Corporation, Ann Arbor, Michigan 48 105). These consensus sequences were assembled. The contig 2511785 overlaps with contig 3506132, and this new contig was called 2535 (SEQ ID NO:27). The contigs from the Sequencher program were copied into the Sequence Analysis software package of GCG.

Each contig was translated in all six reading frames into protein sequences. The *M. alpina*  $\Delta 5$  (MA29) and  $\Delta 6$  (MA524) sequences were compared with each of the translated contigs using the FastA search (a Pearson and Lipman search for similarity between a query sequence and a group of sequences of the same type (nucleic acid or protein)). Homology among these sequences suggest the open reading frames of each contig. The homology among the *M. alpina*  $\Delta 5$  and  $\Delta 6$  to contigs 2535 and 3854933 were utilized to create the final contig called 253538a. Figure 13 is the FastA match of the final contig 253538a and MA29, and Figure 14 is the FastA match of the final contig 253538a and MA524. The DNA sequences for the various contigs are presented in SEQ ID NO:21 -SEQ ID NO:27. The various peptide sequences are shown in SEQ ID NO:28 - SEQ ID NO:34.

Although the open reading frame was generated by merging the two contigs, the contig 2535 shows that there is a unique sequence in the beginning of this contig which does not match with the contig 3854933. Therefore, it is possible that these contigs were generated from independent desaturase like human genes.

The contig 253538a contains an open reading frame encoding 432 amino acids. It starts with Gln (CAG) and ends with the stop codon (TGA). The contig 253538a aligns with both *M. alpina*  $\Delta 5$  and  $\Delta 6$  sequences, suggesting that it could be either of the desaturases, as well as other known desaturases which share homology with each other. The individual contigs listed in Table 6, as well as the intermediate contig 2535 and the final contig 253538a can be utilized to isolate the complete genes for human desaturases.

#### Uses of the human desaturases

These human sequences can be expressed in yeast and plants utilizing the procedures described in the preceding examples. For expression in mammalian cells and transgenic animals, these genes may provide superior codon bias. These human sequences can also be used to identify related desaturase sequences.

Table 7

| Sections of the Desaturases | Clone ID from LifeSeq Database | Keyword               |
|-----------------------------|--------------------------------|-----------------------|
| 151-300 $\Delta 5$          | 3808675                        | Fatty acid desaturase |
| 301-446 $\Delta 5$          | 354535                         | $\Delta 6$            |
| 151-300 $\Delta 6$          | 3448789                        | $\Delta 6$            |
| 151-300 $\Delta 6$          | 1362863                        | $\Delta 6$            |
| 151-300 $\Delta 6$          | 2394760                        | $\Delta 6$            |
| 301-457 $\Delta 6$          | 3350263                        | $\Delta 6$            |

#### Example 10

##### Production of Oil in Microorganisms

The sequences presented in the Sequence Listing including sequences that are homologous to these sequences as well as those sequences that are related to these sequences may be cloned into an appropriate vector using procedures described herein and detailed in the previous examples. Such host cells can be utilized for the production of lipids and/or fats.

Extraction of lipids or fats from host cells follows the general procedure described by Folch, et al., J.Biol. Chem. 226: 497 1957 which is hereby incorporated by reference. Host cells are grown under appropriate conditions and

harvested. Harvested cells are homogenized mechanically with 20ml/g of a mixture of chloroform-methanol, 2:1 (v/v). In order to minimize oxidation, and anti-oxidant is added to the extraction solvent. If the oil is to be utilized for human consumption, vitamin E ( $\alpha$ -tocopherol) is utilized as the anti-oxidant. After  
5 extraction, the solvent containing the lipid extract is washed with 0.2 volume of 0.58% NaCl. The mixture is thoroughly mixed and then transferred to a separating funnel. The mixed material is allowed to settle and two phases are separated out on standing. The bottom phase is collected and the solvent removed under vacuum. The residue contains the total tissue lipids.

10

### **Yeast Strains Rich in Lipids**

Under some circumstances, vectors will be transformed into yeast strains rich in lipids. In these strains, a high percentage of the cell lipids is often stored in fat droplets containing mostly the triglycerides. For these cells, simply breaking  
15 the cell wall by mechanical, pressure or enzymatic means will release the fat droplet. Once released, the fat is extracted with hexane for further processing.

### **Example 12**

#### **Further Processing of Oil Produced by Microorganisms**

20 Further processing of oil produced by microorganisms is necessary to produce an edible food product. The refining process includes the following four steps.

25 1. *Degumming*: Degumming is utilized to remove phosphatides co-extracted with the oil, which tend to separate from the oil as a sludge during storage. Two steps are utilized: (1) phosphatides are precipitated with water and then (2) with a mild acid. The particles are removed by centrifugation.

2. *Alkali Refining*: This step is utilized to further reduce the phosphatide content and also the free fatty acid content of the oil. In this step, the oil is first contacted with small volume of concentrated phosphoric acid in a mixer to precipitate phosphatides. The acidified mixture is then contacted with an aqueous solution of sodium hydroxide to neutralize the free fatty acids as well as any excess phosphoric acid to form a soapy phase. The soap is removed by centrifugation.

3. *Adsorptive Bleaching*: After the alkali-refining step, the crude oil often contains traces of soap and chlorophylloid compounds. The later gives an undesirable green color to the oil and should be removed. An acid-catalyzed clay or active carbon is utilized to remove these compounds.

4. *Deodorization*: The final refining step is deodorization. Deodorization is utilized to remove compounds from the oil that impart undesirable odors and tastes typical of the host organism. The step involves steam distillation of the odor and flavor compounds and other volatile compounds such as free fatty acids from the oil. The oil is heated to 225-260°C under very low pressure to exclude air and the volatile compounds are removed. Deodorization can remove tocopherols to some extent. As the transgenic oil is more unsaturated than non-transgenic oil, additional amount of antioxidant(s) may be supplemented to protect the oil from oxidation.

### Example 13

#### Further Purification of Oil Produced by Microorganisms

Under certain circumstances, further purification and enriching of long-chain polyunsaturated fatty acids from transgenic microorganisms is needed. Such further processing steps include the following:

1. *Fractionation*: Oils are mixtures of triglycerides with different fatty acid compositions. Based on their different melting points, the triglycerides may be separated into different portions. Thermo-mechanical separation processes include

distillation and crystallization. Distillation is generally commercially unsuitable, because the triglyceride mixtures have low vapor pressures and are unstable at high temperature. Crystallization (winterization) uses low temperature to separate solid triglyceride fraction from the liquid fraction. The liquid fraction is usually more  
5 unsaturated than the solid fraction. This method is a mild procedure especially suitable for triglyceride containing the polyunsaturated fatty acids.

2. *Urea fractionation*: Saturated fatty acids generally more readily form stable complexes than do unsaturated fatty acids with urea. The procedure can be used to  
10 enrich polyunsaturated fatty acids.

3. *HPLC separation*: HPLC using a reverse phase column can separate triglyceride molecules according to combined chain length of the fatty acid residues as well as the degree of unsaturation. Using this method, the highly unsaturated triglyceride  
15 molecules are eluted out the column for separation.

4. *Supercritical fluid chromatography (SFC) or supercritical fluid extraction (SFC)*: SFC is a method whereby a highly compressed gas at a temperature and pressure above its critical temperature and pressure as a supercritical fluid is used  
20 to elute analytes from a chromatographic column. Triglycerides are separated by SFC according to the combined chain length of the fatty acid residues using columns coated with relatively non-polar stationary phase, or according to the degree of unsaturation by using a more polar stationary phase. Generally, the triglyceride molecules are separated (1) according to their carbon numbers and (2)  
25 according to unsaturation within each carbon number group. In order words, the most unsaturated molecules have the strongest retention to the stationary phase and are eluted out the column last.

#### **Example 14**

#### **Manufacture of Transgenic Oil Nutritional Products**



Once purified, transgenic oil is incorporated into various food products. The incorporation of transgenic oil into food products involves the following steps: (1) Preparation of slurry mix; (2) blending and (3) standardization:

**I. Preparation of slurry mix:**

5                    Basically, three different slurries are prepared before the final blending.

*A. Protein-in-fat slurry (PIF)*

10                    Add the indicated amounts of oil blend (containing the transgenic canola oil and other oils at a designated ratio) to a kettle and heat at 120-140°F under moderate agitation. This is followed by the addition of DEK Premix, vitamin A, and soy lecithin, and Milei 75L WPC. Maintain the PIF at 120-140°F under moderate agitation until use.

*B. Carbohydrate/mineral slurry (CHO/MIN)*

15                    Heat the required amount of water to 140-160°F with high agitation. Add in order the indicated amounts of TM premix, Gum Arabic, and Fibrim 300, respectively. Allow each addition to be completely dispersed under high agitation before the next ingredient is added.

20                    Add in order the indicated amounts of different mineral salts, dry blend of Gellan Gum and sucrose, and fructooligosaccharides and maltrins. Allow slurry to mix under high agitation until dispersed completely. Maintain the CHO/MIN slurry at 140-160°F under moderate agitation until use.

25                    *C. Protein-in-water slurry (PIW)*

                      Add the indicated amount of water to a kettle and heat to 140-160°F. Add the Alanates, mix under high agitation until protein is completely dispersed,

then slow the agitation to moderate speed. Maintain PIW under moderate agitation at 130-150°F for at least half hour before blending.

## **II. Blending:**

- 5                   Weigh the indicated amount of PIF slurry and add to the PIW slurry, and mix under moderate agitation for 5 min. Add the CHO/MIN to the blend, and adjust to the designated pH value with 1N KOH. Thereafter, deaerate the mix by vacuum, heat the mixture to 160-180°F, and emulsify mix at
- 10                   treatment, homogenized and pass the mix through a holding tube, and then cool mix to 34-45°F and store until standardization.

## **III. Standardization:**

- 15                   Add indicated amount water, vitamin solution, flavor solution, and color solution to the batch. Allow to mix thoroughly. Transfer into can, sterilize and store until use.

### **Example 15**

#### **Nutritional Compositions**

- 20                   The PUFAs of the previous examples can be utilized in various nutritional supplements, infant formulations, nutritional substitutes and other nutrition solutions.

## I. INFANT FORMULATIONS

### A. Isomil® Soy Formula with Iron.

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cow's milk. A feeding for patients with disorders for which lactose should be avoided: lactase deficiency, lactose intolerance and galactosemia.

#### Features:

- Soy protein isolate to avoid symptoms of cow's-milk-protein allergy or sensitivity
- Lactose-free formulation to avoid lactose-associated diarrhea
- Low osmolality (240 mOsm/kg water) to reduce risk of osmotic diarrhea.
- Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.
- 1.8 mg of Iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- Recommended levels of vitamins and minerals.
- Vegetable oils to provide recommended levels of essential fatty acids.
- Milk-white color, milk-like consistency and pleasant aroma.

Ingredients: (Pareve) 85% water, 4.9% corn syrup, 2.6% sugar (sucrose), 2.1% soy oil, 1.9% soy protein isolate, 1.4% coconut oil, 0.15% calcium citrate, 0.11 % calcium phosphate tribasic, potassium citrate, potassium phosphate monobasic, potassium chloride, mono- and diglycerides, soy lecithin, carrageenan, ascorbic acid, L-methionine, magnesium chloride, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D<sub>3</sub> and cyanocobalamin.

**B. Isomil® DF Soy Formula For Diarrhea.**

Usage: As a short-term feeding for the dietary management of diarrhea in infants and toddlers.

**Features:**

- 5
  - First infant formula to contain added dietary fiber from soy fiber specifically for diarrhea management.
  - Clinically shown to reduce the duration of loose, watery stools during mild to severe diarrhea in infants.
  - Nutritionally complete to meet the nutritional needs of the infant.
- 10
  - Soy protein isolate with added L-methionine meets or exceeds an infant's requirement for all essential amino acids.
  - Lactose-free formulation to avoid lactose-associated diarrhea.
  - Low osmolality (240 mOsm/kg water) to reduce the risk of osmotic diarrhea.
- 15
  - Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.
  - Meets or exceeds the vitamin and mineral levels recommended by the Committee on Nutrition of the American Academy of Pediatrics and
- 20
  - required by the Infant Formula Act.
  - 1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
  - Vegetable oils to provide recommended levels of essential fatty acids.

25      **Ingredients:** (Pareve) 86% water, 4.8% corn syrup, 2.5% sugar (sucrose), 2.1% soy oil, 2.0% soy protein isolate, 1.4% coconut oil, 0.77% soy fiber, 0.12% calcium citrate, 0.11 % calcium phosphate tribasic, 0.10% potassium citrate, potassium chloride, potassium phosphate monobasic, mono- and diglycerides, soy lecithin, carrageenan, magnesium chloride, ascorbic acid, L-methionine, potassium

phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phyloquinone, biotin, sodium selenite, vitamin D<sub>3</sub> and cyanocobalamin.

**C. Isomil® SF Sucrose-Free Soy Formula With Iron.**

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cow's-milk protein or an intolerance to sucrose. A feeding for patients with disorders for which lactose and sucrose should be avoided.

10 Features:

- Soy protein isolate to avoid symptoms of cow's-milk-protein allergy or sensitivity.

- Lactose-free formulation to avoid lactose-associated diarrhea (carbohydrate source is Polycose® Glucose Polymers).

15 • Sucrose free for the patient who cannot tolerate sucrose.

- Low osmolality (180 mOsm/kg water) to reduce risk of osmotic diarrhea.

- 1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.

20 • Recommended levels of vitamins and minerals.

- Vegetable oils to provide recommended levels of essential fatty acids.

- Milk-white color, milk-like consistency and pleasant aroma.

Ingredients: (Pareve) 75% water, 11.8% hydrolyzed cornstarch, 4.1% soy oil, 4.1% soy protein isolate, 2.8% coconut oil, 1.0% modified cornstarch, 0.38% calcium phosphate tribasic, 0.17% potassium citrate, 0.13% potassium chloride, 25 mono- and diglycerides, soy lecithin, magnesium chloride, ascorbic acid, L-methionine, calcium carbonate, sodium chloride, choline chloride, carrageenan, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate,

thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D<sub>3</sub> and cyanocobalamin.

5                   **D.     Isomil® 20 Soy Formula With Iron Ready To Feed,  
                    20 Cal/fl oz.**

Usage: When a soy feeding is desired.

Ingredients: (Pareve) 85% water, 4.9% corn syrup, 2.6% sugar (sucrose), 2.1% soy oil, 1.9% soy protein isolate, 1.4% coconut oil, 0.15% calcium citrate, 0.11% calcium phosphate tribasic, potassium citrate, potassium phosphate  
10           monobasic, potassium chloride, mono- and diglycerides, soy lecithin, carrageenan, ascorbic acid, L-methionine, magnesium chloride, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride,  
15           riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D<sub>3</sub> and cyanocobalamin.

**E.     Similac® Infant Formula**

Usage: When an infant formula is needed: if the decision is made to  
discontinue breastfeeding before age 1 year, if a supplement to breastfeeding is  
20           needed or as a routine feeding if breastfeeding is not adopted.

Features:

- Protein of appropriate quality and quantity for good growth; heat-denatured, which reduces the risk of milk-associated enteric blood loss.
- Fat from a blend of vegetable oils (doubly homogenized), providing  
25           essential linoleic acid that is easily absorbed.
- Carbohydrate as lactose in proportion similar to that of human milk.
- Low renal solute load to minimize stress on developing organs.
- Powder, Concentrated Liquid and Ready To Feed forms.

Ingredients: Water, nonfat milk, lactose, soy oil, coconut oil, mono- and diglycerides, soy lecithin, ascorbic acid, carrageenan, choline chloride, taurine, m-inositol, alpha-tocopheryl acetate, zinc sulfate, niacinamid, ferrous sulfate, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium selenite, vitamin D<sub>3</sub> and cyanocobalamin.

#### F. Similac® NeoCare Premature Infant Formula With Iron

Usage: For premature infants' special nutritional needs after hospital discharge. Similac NeoCare is a nutritionally complete formula developed to provide premature infants with extra calories, protein, vitamins and minerals needed to promote catch-up growth and support development.

##### Features:

- Reduces the need for caloric and vitamin supplementation. More calories (22 Cal/fl oz) than standard term formulas (20 Cal/fl oz).
- Highly absorbed fat blend, with medium-chain triglycerides (MCT oil) to help meet the special digestive needs of premature infants.
- Higher levels of protein, vitamins and minerals per 100 Calories to extend the nutritional support initiated in-hospital.
- More calcium and phosphorus for improved bone mineralization.

Ingredients: Corn syrup solids, nonfat milk, lactose, whey protein concentrate, soy oil, high-oleic safflower oil, fractionated coconut oil (medium-chain triglycerides), coconut oil, potassium citrate, calcium phosphate tribasic, calcium carbonate, ascorbic acid, magnesium chloride, potassium chloride, sodium chloride, taurine, ferrous sulfate, m-inositol, choline chloride, ascorbyl palmitate, L-carnitine, alpha-tocopheryl acetate, zinc sulfate, niacinamide, mixed tocopherols, sodium citrate, calcium pantothenate, cupric sulfate, thiamine chloride hydrochloride, vitamin A palmitate, beta carotene, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium selenite, vitamin D<sub>3</sub> and cyanocobalamin.

**G. Similac Natural Care Low-Iron Human Milk Fortifier Ready To Use, 24 Cal/fl oz.**

Usage: Designed to be mixed with human milk or to be fed alternatively with human milk to low-birth-weight infants.

5           Ingredients: Water, nonfat milk, hydrolyzed cornstarch, lactose, fractionated coconut oil (medium-chain triglycerides), whey protein concentrate, soil oil, coconut oil, calcium phosphate tribasic, potassium citrate, magnesium chloride, sodium citrate, ascorbic acid, calcium carbonate, mono- and diglycerides, soy lecithin, carrageenan, choline chloride, m-inositol, taurine, niacinamide, L-  
10           carnitine, alpha tocopheryl acetate, zinc sulfate, potassium chloride, calcium pantothenate, ferrous sulfate, cupric sulfate, riboflavin, vitamin A palmitate, thiamine chloride hydrochloride, pyridoxine hydrochloride, biotin, folic acid, manganese sulfate, phylloquinone, vitamin D<sub>3</sub>, sodium selenite and cyanocobalamin.

15           Various PUFAs of this invention can be substituted and/or added to the infant formulae described above and to other infant formulae known to those in the art..

## **II. NUTRITIONAL FORMULATIONS**

### **A. ENSURE®**

20           Usage: ENSURE is a low-residue liquid food designed primarily as an oral nutritional supplement to be used with or between meals or, in appropriate amounts, as a meal replacement. ENSURE is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets. Although it is primarily an oral supplement, it can be fed by tube.

#### **25           Patient Conditions:**

- For patients on modified diets
- For elderly patients at nutrition risk
- For patients with involuntary weight loss
- For patients recovering from illness or surgery



- For patients who need a low-residue diet

**Ingredients:**

Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Gellan Gum, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Sodium Molybdate, Chromium Chloride, Biotin, Potassium Iodide, Sodium Selenate.

**B. ENSURE® BARS**

Usage: ENSURE BARS are complete, balanced nutrition for supplemental use between or with meals. They provide a delicious, nutrient-rich alternative to other snacks. ENSURE BARS contain <1 g lactose/bar, and Chocolate Fudge Brownie flavor is gluten-free. (Honey Graham Crunch flavor contains gluten.)

**Patient Conditions:**

- For patients who need extra calories, protein, vitamins and minerals
- Especially useful for people who do not take in enough calories and nutrients
- For people who have the ability to chew and swallow
- Not to be used by anyone with a peanut allergy or any type of allergy to nuts.

**Ingredients:**

Honey Graham Crunch -- High-Fructose Corn Syrup, Soy Protein Isolate, Brown Sugar, Honey, Maltodextrin (Corn), Crisp Rice (Milled Rice, Sugar [Sucrose], Salt [Sodium Chloride] and Malt), Oat Bran, Partially Hydrogenated Cottonseed and Soy Oils, Soy Polysaccharide, Glycerine, Whey Protein Concentrate, Polydextrose, Fructose, Calcium Caseinate, Cocoa Powder, Artificial

Flavors, Canola Oil, High-Oleic Safflower Oil, Nonfat Dry Milk, Whey Powder, Soy Lecithin and Corn Oil. Manufactured in a facility that processes nuts.

**Vitamins and Minerals:**

5 Calcium Phosphate Tribasic, Potassium Phosphate Dibasic, Magnesium Oxide, Salt (Sodium Chloride), Potassium Chloride, Ascorbic Acid, Ferric Orthophosphate, Alpha-Tocopheryl Acetate, Niacinamide, Zinc Oxide, Calcium Pantothenate, Copper Gluconate, Manganese Sulfate, Riboflavin, Beta-Carotene, Pyridoxine Hydrochloride, Thiamine Mononitrate, Folic Acid, Biotin, Chromium Chloride, Potassium Iodide, Sodium Selenate, Sodium Molybdate, Phylloquinone,  
10 Vitamin D<sub>3</sub> and Cyanocobalamin.

**Protein:**

**Honey Graham Crunch** - The protein source is a blend of soy protein isolate and milk proteins.

|    |                     |     |
|----|---------------------|-----|
| 15 | Soy protein isolate | 74% |
|    | Milk proteins       | 26% |

**Fat:**

Honey Graham Crunch - The fat source is a blend of partially hydrogenated cottonseed and soybean, canola, high oleic safflower, and corn oils, and soy lecithin.

|    |   |     |
|----|---|-----|
| 20 | Partially hydrogenated cottonseed and soybean oil | 76% |
|    | Canola oil  | 8%  |
|    | High-oleic safflower oil                          | 8%  |
|    | Corn oil  | 4%  |
|    | Soy lecithin                                      | 4%  |

25 **Carbohydrate:**

Honey Graham Crunch - The carbohydrate source is a combination of high-fructose corn syrup, brown sugar, maltodextrin, honey, crisp rice, glycerine, soy polysaccharide, and oat bran.

|   |                          |     |
|---|--------------------------|-----|
|   | High-fructose corn syrup | 24% |
|   | Brown sugar              | 21% |
|   | Maltodextrin             | 12% |
|   | Honey                    | 11% |
| 5 | Crisp rice               | 9%  |
|   | Glycerine                | 9%  |
|   | Soy polysaccharide       | 7%  |
|   | Oat bran                 | 7%  |

## 10 C. ENSURE® HIGH PROTEIN

Usage: ENSURE HIGH PROTEIN is a concentrated, high-protein liquid food designed for people who require additional calories, protein, vitamins, and minerals in their diets. It can be used as an oral nutritional supplement with or between meals or, in appropriate amounts, as a meal replacement. ENSURE  
 15 HIGH PROTEIN is lactose- and gluten-free, and is suitable for use by people recovering from general surgery or hip fractures and by patients at risk for pressure ulcers.

### Patient Conditions

- For patients who require additional calories, protein, vitamins, and minerals,  
 20 such as patients recovering from general surgery or hip fractures, patients at risk for pressure ulcers, and patients on low-cholesterol diets

### Features-

- Low in saturated fat
- Contains 6 g of total fat and < 5 mg of cholesterol per serving
- 25 • Rich, creamy taste
- Excellent source of protein, calcium, and other essential vitamins and minerals
- For low-cholesterol diets
- Lactose-free, easily digested

**Ingredients:**

**Vanilla Supreme:** Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Gellan Gum, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Sodium Motybdate, Chromium Chloride, Biotin, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D.3 and Cyanocobalamin.

**Protein:**

The protein source is a blend of two high-biologic-value proteins: casein and soy.

|    |                               |     |
|----|-------------------------------|-----|
|    | Sodium and calcium caseinates | 85% |
| 15 | Soy protein isolate           | 15% |

**Fat:**

The fat source is a blend of three oils: high-oleic safflower, canola, and soy.

|    |                          |     |
|----|--------------------------|-----|
|    | High-oleic safflower oil | 40% |
|    | Canola oil               | 30% |
| 20 | Soy oil                  | 30% |

The level of fat in ENSURE HIGH PROTEIN meets American Heart Association (AHA) guidelines. The 6 grams of fat in ENSURE HIGH PROTEIN represent 24% of the total calories, with 2.6% of the fat being from saturated fatty acids and 7.9% from polyunsaturated fatty acids. These values are within the AHA guidelines of  $\leq 30\%$  of total calories from fat,  $< 10\%$  of the calories from saturated fatty acids, and  $\leq 10\%$  of total calories from polyunsaturated fatty acids.

**Carbohydrate:**

ENSURE HIGH PROTEIN contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla supreme, chocolate royal,

wild berry, and banana), plus VARI-FLAVORSO® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

#### **Vanilla and other nonchocolate flavors**

|   |              |     |
|---|--------------|-----|
| 5 | Sucrose      | 60% |
|   | Maltodextrin | 40% |

#### **Chocolate**

|  |              |     |
|--|--------------|-----|
|  | Sucrose      | 70% |
|  | Maltodextrin | 30% |

10

#### **D. ENSURE® LIGHT**

Usage: ENSURE LIGHT is a low-fat liquid food designed for use as an oral nutritional supplement with or between meals. ENSURE LIGHT is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

15

#### **Patient Conditions:**

- For normal-weight or overweight patients who need extra nutrition in a supplement that contains 50% less fat and 20% fewer calories than ENSURE
- For healthy adults who don't eat right and need extra nutrition

20

#### **Features:**

- Low in fat and saturated fat
- Contains 3 g of total fat per serving and < 5 mg cholesterol
- Rich, creamy taste
- Excellent source of calcium and other essential vitamins and minerals
- For low-cholesterol diets
- Lactose-free, easily digested

25

**Ingredients:**

**French Vanilla:** Water, Maltodextrin (Corn), Sugar (Sucrose), Calcium Caseinate, High-Oleic Safflower Oil, Canola Oil, Magnesium Chloride, Sodium Citrate, Potassium Citrate, Potassium Phosphate Dibasic, Magnesium Phosphate Dibasic, Natural and Artificial Flavor, Calcium Phosphate Tribasic, Cellulose Gel, Choline Chloride, Soy Lecithin, Carrageenan, Salt (Sodium Chloride), Ascorbic Acid, Cellulose Gum, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Zinc Sulfate, Niacinamide, Manganese Sulfate, Calcium Pantothenate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Vitamin A Palmitate, Pyridoxine Hydrochloride, Riboflavin, Chromium Chloride, Folic Acid, Sodium Molybdate, Biotin, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D<sub>3</sub> and Cyanocobalamin.

**Protein:**

The protein source is calcium caseinate.

|                   |      |
|-------------------|------|
| Calcium caseinate | 100% |
|-------------------|------|

**15 Fat**

The fat source is a blend of two oils: high-oleic safflower and canola.

|                          |     |
|--------------------------|-----|
| High-oleic safflower oil | 70% |
|--------------------------|-----|

|            |     |
|------------|-----|
| Canola oil | 30% |
|------------|-----|

The level of fat in ENSURE LIGHT meets American Heart Association (AHA) guidelines. The 3 grams of fat in ENSURE LIGHT represent 13.5% of the total calories, with 1.4% of the fat being from saturated fatty acids and 2.6% from polyunsaturated fatty acids. These values are within the AHA guidelines of  $\leq 30\%$  of total calories from fat,  $< 10\%$  of the calories from saturated fatty acids, and  $\leq 10\%$  of total calories from polyunsaturated fatty acids.

**25 Carbohydrate**

ENSURE LIGHT contains a combination of maltodextrin and sucrose. The chocolate flavor contains corn syrup as well. The mild sweetness and flavor variety (French vanilla, chocolate supreme, strawberry swirl), plus VARI-

FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

**Vanilla and other nonchocolate flavors**

|   |              |     |
|---|--------------|-----|
|   | Sucrose      | 51% |
| 5 | Maltodextrin | 49% |

**Chocolate**

|  |              |       |
|--|--------------|-------|
|  | Sucrose      | 47.0% |
|  | Corn Syrup   | 26.5% |
|  | Maltodextrin | 26.5% |

10 **Vitamins and Minerals**

An 8-fl-oz serving of ENSURE LIGHT provides at least 25% of the RDIs for 24 key vitamins and minerals.

**Caffeine**

Chocolate flavor contains 2.1 mg caffeine/8 fl oz.

15

**E. ENSURE PLUS®**

Usage: ENSURE PLUS is a high-calorie, low-residue liquid food for use when extra calories and nutrients, but a normal concentration of protein, are needed. It is designed primarily as an oral nutritional supplement to be used with or between meals or, in appropriate amounts, as a meal replacement. ENSURE PLUS is lactose- and gluten-free. Although it is primarily an oral nutritional supplement, it can be fed by tube.

20

**Patient Conditions:**

- For patients who require extra calories and nutrients, but a normal concentration of protein, in a limited volume
- For patients who need to gain or maintain healthy weight

25

**Features**

- Rich, creamy taste
- Good source of essential vitamins and minerals

**Ingredients**

- 5 **Vanilla:** Water, Corn Syrup, Maltodextrin (Corn), Corn Oil, Sodium and Calcium Caseinates, Sugar (Sucrose), Soy Protein Isolate, Magnesium Chloride, Potassium Citrate, Calcium Phosphate Tribasic, Soy Lecithin, Natural and Artificial Flavor, Sodium Citrate, Potassium Chloride, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate,
- 10 Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Chromium Chloride, Sodium Molybdate, Potassium Iodide, Sodium Selenite, Phylloquinone, Cyanocobalamin and Vitamin D<sub>3</sub>.

**Protein**

- 15 The protein source is a blend of two high-biologic-value proteins: casein and soy.

|                               |     |
|-------------------------------|-----|
| Sodium and calcium caseinates | 84% |
| Soy protein isolate           | 16% |

**Fat**

- 20 The fat source is corn oil.

|          |      |
|----------|------|
| Corn oil | 100% |
|----------|------|

**Carbohydrate**

- 25 ENSURE PLUS contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, strawberry, coffee, butter pecan, and eggnog), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

**Vanilla, strawberry, butter pecan, and coffee flavors**



|              |     |
|--------------|-----|
| Corn Syrup   | 39% |
| Maltodextrin | 38% |
| Sucrose      | 23% |

#### **Chocolate and eggnog flavors**

|   |              |     |
|---|--------------|-----|
| 5 | Corn Syrup   | 36% |
|   | Maltodextrin | 34% |
|   | Sucrose      | 30% |

#### **Vitamins and Minerals**

10 An 8-fl-oz serving of ENSURE PLUS provides at least 15% of the RDIs for 25 key Vitamins and minerals.

#### **Caffeine**

Chocolate flavor contains 3.1 mg Caffeine/8 fl oz. Coffee flavor contains a trace amount of caffeine.

### 15 **F. ENSURE PLUS® HN**

Usage: ENSURE PLUS HN is a nutritionally complete high-calorie, high-nitrogen liquid food designed for people with higher calorie and protein needs or limited volume tolerance. It may be used for oral supplementation or for total nutritional support by tube. ENSURE PLUS HN is lactose- and gluten-free.

### 20 **Patient Conditions:**

- For patients with increased calorie and protein needs, such as following surgery or injury
- For patients with limited volume tolerance and early satiety

#### **Features**

- 25
- For supplemental or total nutrition
  - For oral or tube feeding

- 1.5 CaV/mL
- High nitrogen
- Calorically dense

**Ingredients**

- 5 **Vanilla:** Water, Maltodextrin (Corn), Sodium and Calcium Caseinates, Corn Oil, Sugar (Sucrose), Soy Protein Isolate, Magnesium Chloride, Potassium Citrate, Calcium Phosphate Tribasic, Soy Lecithin, Natural and Artificial Flavor, Sodium Citrate, Choline Chloride, Ascorbic Acid, Taurine, L-Carnitine, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Carrageenan, Calcium
- 10 Pantothenate, Manganese Sulfate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Chromium Chloride, Sodium Molybdate, Potassium Iodide, Sodium Selenite, Phylloquinone, Cyanocobalamin and Vitamin D<sub>3</sub>.

15 **G. ENSURE® POWDER**

Usage: ENSURE POWDER (reconstituted with water) is a low-residue liquid food designed primarily as an oral nutritional supplement to be used with or between meals. ENSURE POWDER is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

20 **Patient Conditions:**

- For patients on modified diets
- For elderly patients at nutrition risk
- For patients recovering from illness/surgery
- For patients who need a low-residue diet

25 **Features**

- Convenient, easy to mix
- Low in saturated fat

- Contains 9 g of total fat and < 5 mg of cholesterol per serving
- High in vitamins and minerals
- For low-cholesterol diets
- Lactose-free, easily digested

5     **Ingredients:** Corn Syrup, Maltodextrin (Corn), Sugar (Sucrose), Corn Oil, Sodium and Calcium Caseinates, Soy Protein Isolate, Artificial Flavor, Potassium Citrate, Magnesium Chloride, Sodium Citrate, Calcium Phosphate Tribasic, Potassium Chloride, Soy Lecithin, Ascorbic Acid, Choline Chloride, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Calcium Pantothenate, 10     Manganese Sulfate, Thiamine Chloride Hydrochloride, Cupric Sulfate, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Sodium Molybdate, Chromium Chloride, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D<sub>3</sub> and Cyanocobalamin.

#### **Protein**

15             The protein source is a blend of two high-biologic-value proteins: casein and soy.

|                               |     |
|-------------------------------|-----|
| Sodium and calcium caseinates | 84% |
| Soy protein isolate           | 16% |

#### **Fat**

20             The fat source is corn oil.

|          |      |
|----------|------|
| Corn oil | 100% |
|----------|------|

#### **Carbohydrate**

ENSURE POWDER contains a combination of corn syrup, maltodextrin, and sucrose. The mild sweetness of ENSURE POWDER, plus VARI- 25     FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, helps to prevent flavor fatigue and aid in patient compliance.

#### **Vanilla**

|            |     |
|------------|-----|
| Corn Syrup | 35% |
|------------|-----|

|              |     |
|--------------|-----|
| Maltodextrin | 35% |
| Sucrose      | 30% |

## H. ENSURE® PUDDING

5           Usage: ENSURE PUDDING is a nutrient-dense supplement providing balanced nutrition in a nonliquid form to be used with or between meals. It is appropriate for consistency-modified diets (e.g., soft, pureed, or full liquid) or for people with swallowing impairments. ENSURE PUDDING is gluten-free.

### Patient Conditions:

- 10           • For patients on consistency-modified diets (e.g., soft, pureed, or full liquid)
- For patients with swallowing impairments
- **Features**
- Rich and creamy, good taste
- Good source of essential vitamins and minerals • Convenient-needs no
- 15           refrigeration
- Gluten-free

**Nutrient Profile per 5 oz:** Calories 250, Protein 10.9%, Total Fat 34.9%, Carbohydrate 54.2%

### Ingredients:

- 20           **Vanilla:** Nonfat Milk, Water, Sugar (Sucrose), Partially Hydrogenated Soybean Oil, Modified Food Starch, Magnesium Sulfate. Sodium Stearoyl Lactylate, Sodium Phosphate Dibasic, Artificial Flavor, Ascorbic Acid, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Choline Chloride, Niacinamide, Manganese Sulfate, Calcium Pantothenate, FD&C Yellow #5, Potassium Citrate, Cupric
- 25           Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, FD&C Yellow #6, Folic Acid, Biotin, Phylloquinone, Vitamin D3 and Cyanocobalamin.

### Protein

The protein source is nonfat milk.

Nonfat milk 100%

#### **Fat**

The fat source is hydrogenated soybean oil.

5 Hydrogenated soybean oil 100%

#### **Carbohydrate**

10 ENSURE PUDDING contains a combination of sucrose and modified food starch. The mild sweetness and flavor variety (vanilla, chocolate, butterscotch, and tapioca) help prevent flavor fatigue. The product contains 9.2 grams of lactose per serving.

#### **Vanilla and other nonchocolate flavors**

Sucrose 56%

Lactose 27%

Modified food starch 17%

#### **15 Chocolate**

Sucrose 58%

Lactose 26%

Modified food starch 16%

#### **20 I. ENSURE® WITH FIBER**

Usage: ENSURE WITH FIBER is a fiber-containing, nutritionally complete liquid food designed for people who can benefit from increased dietary fiber and nutrients. ENSURE WITH FIBER is suitable for people who do not require a low-residue diet. It can be fed orally or by tube, and can be used as a nutritional supplement to a regular diet or, in appropriate amounts, as a meal replacement. ENSURE WITH FIBER is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

25

**Patient Conditions**

- For patients who can benefit from increased dietary fiber and nutrients

**Features**

- New advanced formula-low in saturated fat, higher in vitamins and minerals
- 5 • Contains 6 g of total fat and < 5 mg of cholesterol per serving
- Rich, creamy taste
- Good source of fiber
- Excellent source of essential vitamins and minerals
- For low-cholesterol diets
- 10 • Lactose- and gluten-free

**Ingredients**

**Vanilla:** Water, Maltodextrin (Corn), Sugar (Sucrose), Sodium and Calcium Caseinates, Oat Fiber, High-Oleic Safflower Oil, Canola Oil, Soy Protein Isolate, Corn Oil, Soy Fiber, Calcium Phosphate Tribasic, Magnesium Chloride, Potassium Citrate, Cellulose Gel, Soy Lecithin, Potassium Phosphate Dibasic, Sodium Citrate, Natural and Artificial Flavors, Choline Chloride, Magnesium Phosphate, Ascorbic Acid, Cellulose Gum, Potassium Chloride, Carrageenan, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Zinc Sulfate, Niacinamide, Manganese Sulfate, Calcium Pantothenate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Chromium Chloride, Biotin, Sodium Molybdate, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D<sub>3</sub> and Cyanocobalamin.

**Protein**

25 The protein source is a blend of two high-biologic-value proteins- casein and soy.

|                               |     |
|-------------------------------|-----|
| Sodium and calcium caseinates | 80% |
| Soy protein isolate           | 20% |

**Fat**

The fat source is a blend of three oils: high-oleic safflower, canola, and corn.

|   |                          |     |
|---|--------------------------|-----|
|   | High-oleic safflower oil | 40% |
| 5 | Canola oil               | 40% |
|   | Corn oil                 | 20% |

The level of fat in ENSURE WITH FIBER meets American Heart Association (AHA) guidelines. The 6 grams of fat in ENSURE WITH FIBER represent 22% of the total calories, with 2.01 % of the fat being from saturated fatty acids and 6.7% from polyunsaturated fatty acids. These values are within the AHA guidelines of  $\leq 30\%$  of total calories from fat,  $< 10\%$  of the calories from saturated fatty acids, and  $\leq 10\%$  of total calories from polyunsaturated fatty acids.

**Carbohydrate**

ENSURE WITH FIBER contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, and butter pecan), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

**Vanilla and other nonchocolate flavors**

|    |              |     |
|----|--------------|-----|
|    | Maltodextrin | 66% |
| 20 | Sucrose      | 25% |
|    | Oat Fiber    | 7%  |
|    | Soy Fiber    | 2%  |

**Chocolate**

|    |              |     |
|----|--------------|-----|
|    | Maltodextrin | 55% |
| 25 | Sucrose      | 36% |
|    | Oat Fiber    | 7%  |
|    | Soy Fiber    | 2%  |

## Fiber

The fiber blend used in ENSURE WITH FIBER consists of oat fiber and soy polysaccharide. This blend results in approximately 4 grams of total dietary fiber per 8-fl-oz can. The ratio of insoluble to soluble fiber is 95:5.

- 5           The various nutritional supplements described above and known to others of skill in the art can be substituted and/or supplemented with the PUFAs of this invention.

## J. Oxepa™ Nutritional Product

- 10           Oxepa is low-carbohydrate, calorically dense enteral nutritional product designed for the dietary management of patients with or at risk for ARDS. It has a unique combination of ingredients, including a patented oil blend containing eicosapentaenoic acid (EPA from fish oil),  $\gamma$ -linolenic acid (GLA from borage oil), and elevated antioxidant levels.

### Caloric Distribution:

- 15           • Caloric density is high at 1.5 Cal/mL (355 Cal/8 fl oz), to minimize the volume required to meet energy needs.
- The distribution of Calories in Oxepa is shown in Table 7.

| Table 7. Caloric Distribution of Oxepa |              |           |          |
|--|--------------|-----------|----------|
|  | per 8 fl oz. | per liter | % of Cal |
| Calories                               | 355          | 1,500     | ---      |
| Fat (g)                                | 22.2         | 93.7      | 55.2     |
| Carbohydrate (g)                       | 25           | 105.5     | 28.1     |
| Protein (g)                            | 14.8         | 62.5      | 16.7     |
| Water (g)                              | 186          | 785       | ---      |

### Fat:

- 20           • Oxepa contains 22.2 g of fat per 8-fl oz serving (93.7 g/L).
- The fat source is a oil blend of 31.8% canola oil, 25% medium-chain triglycerides (MCTs), 20% borage oil, 20% fish oil, and 3.2 % soy lecithin. The typical fatty acid profile of Oxepa is shown in Table 8.
- 25           • Oxepa provides a balanced amount of polyunsaturated, monounsaturated, and saturated fatty acids, as shown in Table 10.



- Medium-chain triglycerides (MCTs) -- 25% of the fat blend -- aid gastric emptying because they are absorbed by the intestinal tract without emulsification by bile acids.

The various fatty acid components of Oxepa™ nutritional product can be substituted and/or supplemented with the PUFAs of this invention.

5

| Table 8. Typical Fatty Acid Profile |                     |            |       |
|-------------------------------------|---------------------|------------|-------|
|                                     | % Total Fatty Acids | g/8 fl oz* | g/L*  |
| Caproic (6:0)                       | 0.2                 | 0.04       | 0.18  |
| Caprylic (8:0)                      | 14.69               | 3.1        | 13.07 |
| Capric (10:0)                       | 11.06               | 2.33       | 9.87  |
| Palmitic (16:0)                     | 5.59                | 1.18       | 4.98  |
| Palmitoleic (16:1n-7)               | 1.82                | 0.38       | 1.62  |
| Stearic (18:0)                      | 1.84                | 0.39       | 1.64  |
| Oleic (18:1n-9)                     | 24.44               | 5.16       | 21.75 |
| Linoleic (18:2n-6)                  | 16.28               | 3.44       | 14.49 |
| $\alpha$ -Linolenic (18:3n-3)       | 3.47                | 0.73       | 3.09  |
| $\gamma$ -Linolenic (18:3n-6)       | 4.82                | 1.02       | 4.29  |
| Eicosapentaenoic (20:5n-3)          | 5.11                | 1.08       | 4.55  |
| n-3-Docosapentaenoic (22:5n-3)      | 0.55                | 0.12       | 0.49  |
| Docosahexaenoic (22:6n-3)           | 2.27                | 0.48       | 2.02  |
| Others                              | 7.55                | 1.52       | 6.72  |

\* Fatty acids equal approximately 95% of total fat.

| Table 9. Fat Profile of Oxepa. |                              |
|--------------------------------|------------------------------|
| % of total calories from fat   | 55.2                         |
| Polyunsaturated fatty acids    | 31.44 g/L                    |
| Monounsaturated fatty acids    | 25.53 g/L                    |
| Saturated fatty acids          | 32.38 g/L                    |
| n-6 to n-3 ratio               | 1.75:1                       |
| Cholesterol                    | 9.49 mg/8 fl oz<br>40.1 mg/L |

### Carbohydrate:

10

- The carbohydrate content is 25.0 g per 8-fl-oz serving (105.5 g/L).

- The carbohydrate sources are 45% maltodextrin (a complex carbohydrate) and 55% sucrose (a simple sugar), both of which are readily digested and absorbed.
- The high-fat and low-carbohydrate content of Oxepa is designed to minimize carbon dioxide (CO<sub>2</sub>) production. High CO<sub>2</sub> levels can complicate weaning in ventilator-dependent patients. The low level of carbohydrate also may be useful for those patients who have developed stress-induced hyperglycemia.
- Oxepa is lactose-free.

Dietary carbohydrate, the amino acids from protein, and the glycerol moiety of fats can be converted to glucose within the body. Throughout this process, the carbohydrate requirements of glucose-dependent tissues (such as the central nervous system and red blood cells) are met. However, a diet free of carbohydrates can lead to ketosis, excessive catabolism of tissue protein, and loss of fluid and electrolytes. These effects can be prevented by daily ingestion of 50 to 100 g of digestible carbohydrate, if caloric intake is adequate. The carbohydrate level in Oxepa is also sufficient to minimize gluconeogenesis, if energy needs are being met.

**Protein:**

- Oxepa contains 14.8 g of protein per 8-fl-oz serving (62.5 g/L).
- The total calorie/nitrogen ratio (150:1) meets the need of stressed patients.
- Oxepa provides enough protein to promote anabolism and the maintenance of lean body mass without precipitating respiratory problems. High protein intakes are a concern in patients with respiratory insufficiency. Although protein has little effect on CO<sub>2</sub> production, a high protein diet will increase ventilatory drive.
- The protein sources of Oxepa are 86.8% sodium caseinate and 13.2% calcium caseinate.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by

reference to the same extent as if each individual publication or patent application was specifically and individually indicated as incorporated by reference.

5       The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

- 5 1. A transgenic insect cell comprising a nucleotide sequence which encodes a polypeptide wherein the sequence of the polypeptide comprises a sequence selected from the group consisting of residues 30-38, 41-44, 171-175, 203-212 and 387-394 of SEQ ID NO:2.
2. An oil or fraction thereof isolated from the insect cell of claim 1.
- 10 3. A method of treating or preventing malnutrition comprising administering said oil of claim 2 to a patient in need of said treatment or prevention in an amount sufficient to effect said treatment or prevention.
4. A pharmaceutical composition comprising said oil or fraction of claim 2 and a pharmaceutically acceptable carrier.
- 15 5. The pharmaceutical composition of claim 4, wherein said pharmaceutical composition is in the form of a solid or a liquid.
6. The pharmaceutical composition of claim 4, wherein said pharmaceutical composition is in a capsule or tablet form.
- 20 7. The pharmaceutical composition of claim 4 further comprising at least one nutrient selected from the group consisting of a vitamin, a mineral, a carbohydrate, a sugar, an amino acid, a free fatty acid, a phospholipid, an antioxidant, and a phenolic compound.
8. A nutritional formula comprising said oil or fraction thereof of claim 2.
- 25 9. The nutritional formula of claim 8, wherein said nutritional formula is selected from the group consisting of an infant formula, a dietary supplement, and a dietary substitute.

10. The nutritional formula of claim 9, wherein said infant formula, dietary supplement or dietary supplement is in the form of a liquid or a solid.
11. An infant formula comprising said oil or fraction thereof of claim 2.
- 5 12. The infant formula of claim 11 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electro dialysed whey, electro dialysed skim milk, milk whey, soy protein, and other protein hydrolysates.
- 10 13. The infant formula of claim 12 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.
- 15 14. A dietary supplement comprising said oil or fraction thereof of claim 2.
- 15 15. The dietary supplement of claim 14 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electro dialysed whey, electro dialysed skim milk, milk whey, soy protein, and other protein hydrolysates.
- 20 16. The dietary supplement of claim 15 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.
- 25 17. The dietary supplement of claim 16, wherein said dietary supplement is administered to a human or an animal.
- 30 18. A dietary substitute comprising said oil or fraction thereof of claim 2.

19. The dietary substitute of claim 18 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electro dialysed whey, electro dialysed skim milk, milk whey, soy protein, and other protein hydrolysates.
20. The dietary substitute of claim 19 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.
21. The dietary substitute of claim 20, wherein said dietary substitute is administered to a human or animal.
22. A method of treating a patient having a condition caused by insufficient intake or production of polyunsaturated fatty acids comprising administering to said patient said dietary substitute of claim 21 in an amount sufficient to effect said treatment.
23. The method of claim 22, wherein said dietary substitute is administered enterally or parenterally.
24. A cosmetic comprising said oil or fraction thereof of claim 2.
25. The cosmetic of claim 24, wherein said cosmetic is applied topically.
26. The pharmaceutical composition of claim 4, wherein said pharmaceutical composition is administered to a human or an animal.
27. An animal feed comprising said oil or fraction thereof of claim 2.
28. A method for producing an oil or fraction thereof comprising growing one or more transgenic insect cells under suitable conditions whereby said cells express a transgenic polypeptide wherein the sequence of said polypeptide comprises a sequence

selected from the group consisting of residues 30-38, 41-44,  
171-175, 203-212 and 387-394 of SEQ ID NO:2.

- 5
29. An oil or fraction thereof produced from the method of claim 28.
30. A method of treating or preventing malnutrition comprising administering said oil of claim 29 to a patient in need of said treatment or prevention in an amount sufficient to effect said treatment or prevention.
- 10
31. A pharmaceutical composition comprising said oil or fraction of claim 29 and a pharmaceutically acceptable carrier.
32. The pharmaceutical composition of claim 31, wherein said pharmaceutical composition is in the form of a solid or a liquid.
33. The pharmaceutical composition of claim 31, wherein said pharmaceutical composition is in a capsule or tablet form.
- 15
34. The pharmaceutical composition of claim 31 further comprising at least one nutrient selected from the group consisting of a vitamin, a mineral, a carbohydrate, a sugar, an amino acid, a free fatty acid, a phospholipid, an antioxidant, and a phenolic compound.
- 20
35. A nutritional formula comprising said oil or fraction thereof of claim 29.
36. The nutritional formula of claim 35, wherein said nutritional formula is selected from the group consisting of an infant formula, a dietary supplement, and a dietary substitute.
- 25
37. The nutritional formula of claim 36, wherein said infant formula, dietary supplement or dietary supplement is in the form of a liquid or a solid.
38. An infant formula comprising said oil or fraction thereof of claim 29.
39. The infant formula of claim 38 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy

oil, canola oil, mono- and diglycerides, glucose, edible lactose, electrodialysed whey, electrodialysed skim milk, milk whey, soy protein, and other protein hydrolysates.

- 5                   40.   The infant formula of claim 39 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.
- 10                  41.   A dietary supplement comprising said oil or fraction thereof of claim 29.
42.   The dietary supplement of claim 41 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electrodialysed whey, electrodialysed skim milk, milk whey, soy protein, and other protein hydrolysates.
- 15                  43.   The dietary supplement of claim 42 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.
- 20                  44.   The dietary supplement of claim 43, wherein said dietary supplement is administered to a human or an animal.
45.   A dietary substitute comprising said oil or fraction thereof of claim 29.
- 25                  46.   The dietary substitute of claim 45 further comprising at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, mono- and diglycerides, glucose, edible lactose, electrodialysed whey, electrodialysed skim milk, milk whey, soy protein, and other protein hydrolysates.



- 5 47. The dietary substitute of claim 46 further comprising at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex; and at least one mineral selected from the group consisting of calcium, magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium, and iron.
48. The dietary substitute of claim 47, wherein said dietary substitute is administered to a human or animal.
- 10 49. A method of treating a patient having a condition caused by insufficient intake or production of polyunsaturated fatty acids comprising administering to said patient said dietary substitute of claim 52 in an amount sufficient to effect said treatment.
50. The method of claim 49, wherein said dietary substitute or said dietary supplement is administered enterally or parenterally.
51. A cosmetic comprising said oil or fraction thereof of claim 29.
- 15 52. The cosmetic of claim 51, wherein said cosmetic is applied topically.
53. The pharmaceutical composition of claim 31, wherein said pharmaceutical composition is administered to a human or an animal.
- 20 54. An animal feed comprising said oil or fraction thereof of claim 19.
55. A baculovirus vector comprising  
a nucleotide sequence which encodes a polypeptide wherein the sequence of the polypeptide comprises a sequence selected from the group consisting of residues 30-38, 41-44, 171-175, 203-212 and 387-394 of SEQ ID NO:2.
- 25 56. An insect cell comprising the vector of claim 55.
57. The cell of claim 56 wherein said insect is of the genus *Spodoptera*.
58. The cell of the claim 57 wherein said insect is of the species *Frugiperda*.

59. The cell of claim 57 wherein said insect is of the genus *Trichoplusia*.
60. The cell of claim 59 wherein said insect is of the species *ni*.
- 5 61. A method for producing an oil or fraction thereof comprising growing one or more transgenic insect cells under suitable conditions where said cells express a transgenic polypeptide wherein the sequence of said polypeptide comprises a sequence selected from the group consisting of residues 30-38, 41-44, 171-175, 203-212 and 387-394 of SEQ ID NO:2.
- 10 62. The method according to claim 52 including the step of extracting said insect cells with an organic solvent.
63. The method according to claim 51 wherein said oil includes phosphatides and said phosphatides are removed by precipitation with water and with mild acid.
- 15 64. The method according to claim 53 wherein said oil is treated with phosphosphoric acid to precipitate said phosphatides.
65. The method of claim 54 wherein said phosphoric acid is neutralized with sodium hydroxide.
- 20 66. The method of claim 54 wherein said oil is treated with acid catalyzed clay or activated carbon.
67. The method of claim 54 including the further step of deodorizing said oil by subjecting said oil to steam distillation.
- 25 68. A method for producing a  $\Delta 5$  desaturase polypeptide comprising growing one or more transgenic insect cells under suitable conditions where said cells express a transgenic polypeptide wherein the sequence of said polypeptide comprises a sequence selected from the group consisting of residues 30-38, 41-44, 171-175, 203-212 and 387-394 or SEQ ID NO:2.

69. Isolated and purified  $\Delta 5$  desaturase protein purified by the method of claim 68.
70. Antibodies directed to the protein of claim 69.
- 5 71. The antibodies of claim 70 wherein said antibodies are polyclonal antibodies.
72. The antibodies of claim 70 wherein said antibodies are polyclonal antibodies.
- 10 73. A method for production of arachidonic acid comprising growing one or more transgenic insect cells under suitable conditions where said cells express a transgenic polypeptide wherein the sequence of said polypeptide comprises a sequence selected from the group consisting of residues 30-38, 41-44, 171-175, 203-212 and 387-394 of SEQ ID No:2.
- 15 74. Isolated and purified arachidonic acid purified by the method of claim 73.
75. A nutritional supplement comprising the arachidonic acid of claim 74.

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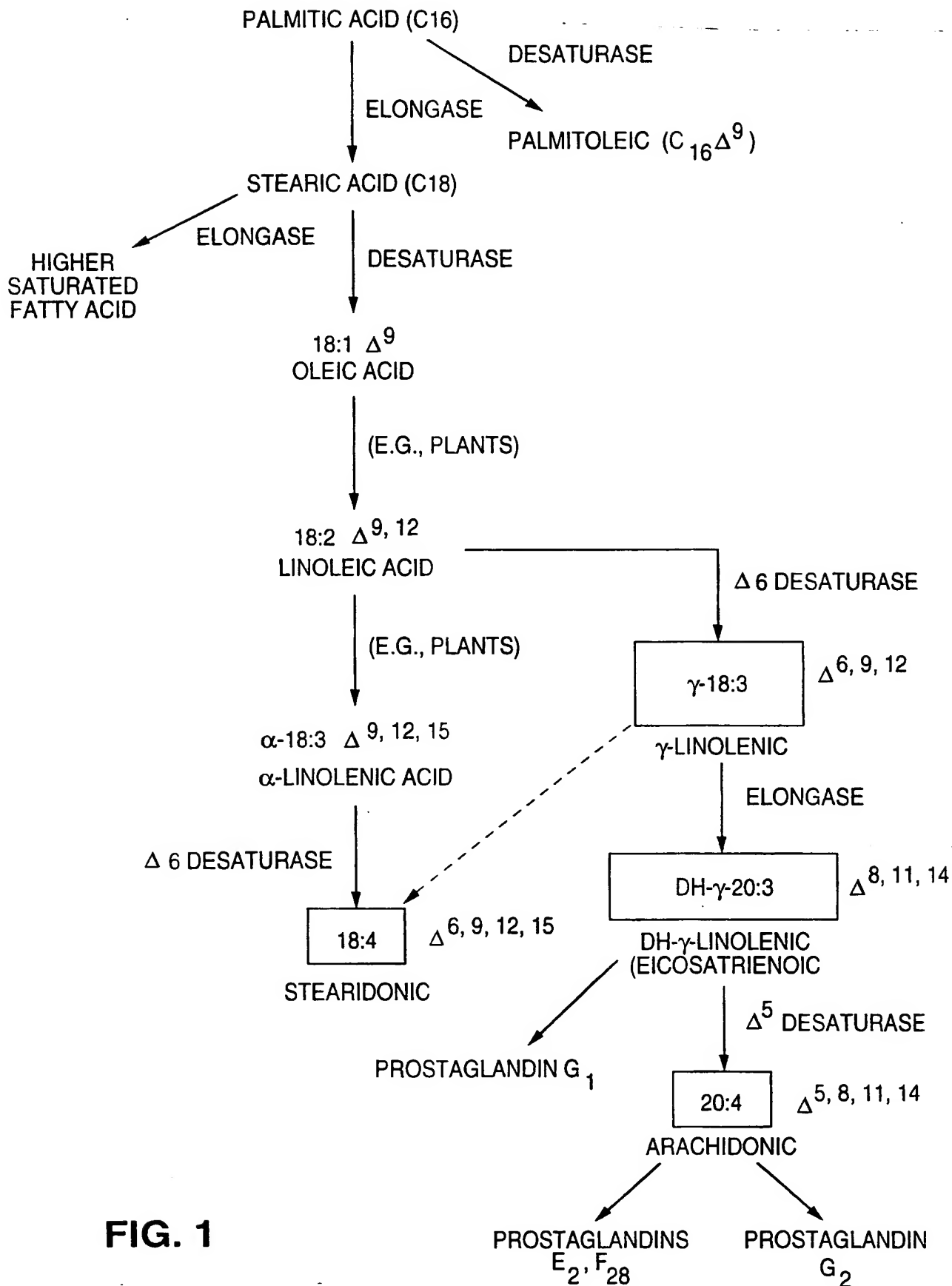


FIG. 1

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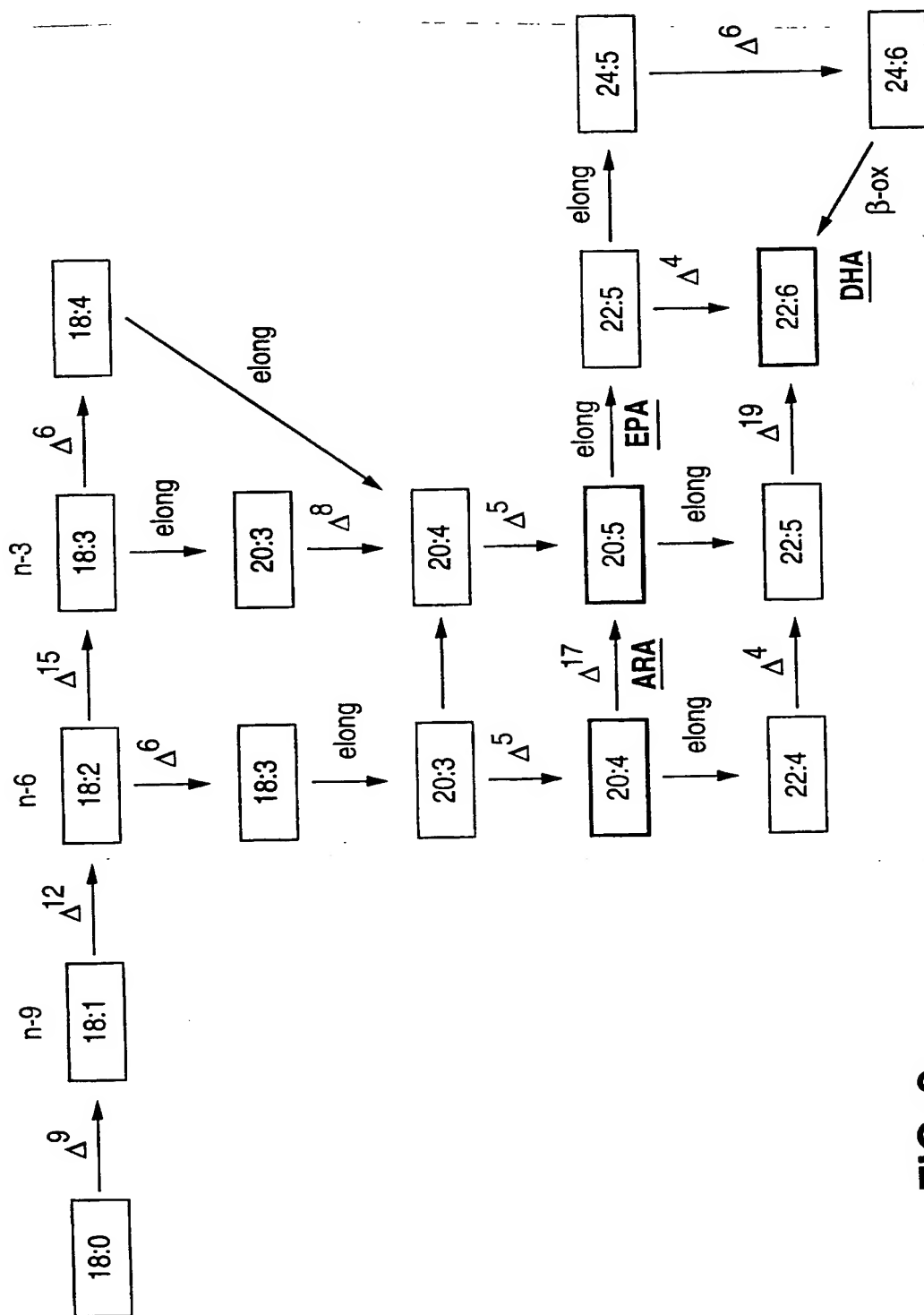


FIG. 2

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GCTTCCTCCA GTTCATCCTC CATTTGGCCA CCTGCA TTCT TTACGACCGT TAAGCAAG
60
ATG GGA ACG GAC CAA GGA AAA ACC TTC ACC TGG GAA GAG CTG GCG GCC
met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Glu Leu Ala Ala
120
CAT AAC ACC AAG GAC GAC CTA CTC TTG GCC ATC CGC GGC AGG GTG TAC
His Asn Thr Lys Asp Asp Leu Leu Leu Ala Ile Arg Gly Arg Val Tyr
180
GAT GTC ACA AAG TTC TTG AGC CGC CAT CCT GGT GGA GTG GAC ACT CTC
Asp Val Thr Lys Phe Leu Ser Arg His Pro Gly Val Asp Thr Leu
240
CTG CTC GGA GCT GGC CGA GAT GTT ACT CCG GTC TTT GAG ATG TAT CAC
Leu Leu Gly Ala Gly Arg Asp Val Thr Pro Val Phe Glu Met Tyr His
300
GCG TTT GGG GCT GCA GAT GCC ATT ATG AAG AAG TAC TAT GTC GGT ACA
Ala Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr
360
CTG GTC TCG AAT GAG CTG CCC ATC TTC CCG GAG CCA ACG GTG TTC CAC
Leu Val Ser Asn Glu Leu Pro Ile Phe Pro Glu Pro Thr Val Phe His
420
AAA ACC ATC AAG ACG AGA GTC GAG GGC TAC TTT ACG GAT CGG AAC ATT
Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Thr Asp Arg Asn Ile

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FIG. 3A

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      420 *
GAT CCC AAG AAT AGA CCA GAG ATC TGG GGA CGA TAC GCT CTT ATC TTT
Asp Pro Lys Asn Arg Arg Pro Glu Ile Trp Gly Arg Tyr Ala Leu Ile Phe

      480 *
GGA TCC TTG ATC GCT TCC TAC TAC GTG GCG CAG CTC TTT GTG CCT TTC GTT
Gly Ser Leu Ile Ile Ala Ala Ser Tyr Tyr Val Phe Val Pro Phe Val

      540 *
GTC GAA CGC ACA TGG CTT CAG GTG GTG TTT GCA ATC ATC ATG GGA TTT
Val Glu Arg Thr Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe

      600 *
GCG TGC GCA CAA GTC GGA CTC AAC CCT CTT CAT GAT GCG TCT CAC TTT
Ala Cys Ala Gln Val Val Gly Leu Asn Pro Leu His Asp Ala Ser His Phe

      660 *
TCA GTG ACC CAC AAC CCC ACT GTC TGG AAG ATT CTG GGA GCC ACG CAC
Ser Val Thr His Asn Asn Thr Val Trp Trp Lys Ile Leu Gly Ala Thr His

      720 *
GAC TTT TTC AAC GGA GCA TCG TAC CTG GTG TGG ATG ATG TAC CAT ATG
Asp Phe Phe Asn Gly Ala Ser Tyr Tyr Leu Val Trp Met Tyr Gln His Met

      780 *
CTC GGC CAT CAC CCC TAC ACC AAC ATT GCT GGA GCA GAT CCC GAC GTG
Leu Gly His His Pro Tyr Thr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val

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FIG. 3B

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TCG  ACG  TCT  GAG  CCC  GAT  GTT  CGT  CTC  ATC  AAG  CCC  AAC  CAA  AAG  TGG
Ser  Thr  Ser  Glu  Pro  Asp  Val  Arg  Arg  Ile  Lys  Pro  Asn  Gln  Lys  Trp
780
TTT  GTC  AAC  CAC  ATC  AAC  CAG  CAC  ATG  TTT  GTT  CCT  TTC  CTG  TAC  GGA
Phe  Val  Asn  His  Ile  Asn  Gln  His  Met  Phe  Val  Pro  Phe  Leu  Tyr  Gly
840
CTG  CTG  GCG  TTC  AAG  GTG  CGC  ATT  CAG  GAC  ATC  AAC  ATT  TTG  TAC  TTT
Leu  Leu  Ala  Phe  Lys  Val  Arg  Ile  Gln  Asp  Ile  Asn  Ile  Leu  Tyr  Phe
900
GTC  AAG  ACC  AAT  GAC  GCT  ATT  CGT  GTC  AAT  CCC  ATC  TCG  ACA  TGG  CAC
Val  Lys  Thr  Asn  Asp  Ala  Ile  Arg  Val  Asn  Pro  Ile  Ser  Thr  Trp  His
960
ACT  GTG  ATG  TTC  TGG  GGC  GGC  AAG  GCT  TTC  TTT  GTC  TGG  TAT  CGC  CTG
Thr  Val  Met  Phe  Trp  Gly  Gly  Lys  Ala  Phe  Phe  Val  Trp  Tyr  Arg  Leu
1020
ATT  GTT  CCC  CTG  CAG  TAT  CTG  CCC  CTG  GGC  AAG  GTG  CTG  CTC  TTG  TTC
Ile  Val  Pro  Leu  Gln  Tyr  Leu  Pro  Leu  Gly  Lys  Val  Leu  Leu  Phe
1080
ACG  GTC  GCG  GAC  ATG  GTG  TCG  TCT  TAC  TGG  CTG  GCG  CTG  ACC  TTC  CAG
Thr  Val  Ala  Asp  Met  Val  Ser  Tyr  Trp  Leu  Leu  Ala  Leu  Thr  Phe  Gln

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FIG. 3C



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1080 \*  
 GCG AAC CAC GTT GTT GAG GAA GTT CAG TGG CCG TTG CCT GAC GAG AAC  
 Ala Asn His Val Val Gln Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn  
 1140 \*  
 GGG ATC ATC CAA AAG GAC TGG GCA GCT ATG CAG GTC GAG ACT ACG CAG  
 Gly Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln  
 1200 \*  
 GAT TAC GCA CAC GAT TCG CAC CTC TGG ACC AGC ATC ACT GGC AGC TTG  
 Asp Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu  
 AAC TAC CAG GCT GTG CAC CAT CTG TTC CCC AAC GTG TCG CAG CAC CAT  
 Asn Tyr Gln Ala Val His His Leu Phe Pro Asn Val Ser Gln His His  
 1260 \*  
 TAT CCC GAT ATT CTG GCC ATC ATC AAG AAC ACC TGC AGC GAG TAC AAG  
 Tyr Pro Asp Ile Leu Ile Ile Lys Asn Thr Cys Ser Glu Tyr Lys  
 1320 \*  
 GTT CCA TAC CTT GTC AAG GAT ACG TTT TGG CAA GCA TTT GCT TCA CAT  
 Val Pro Tyr Leu Val Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His  
 1380 \*  
 TTG GAG CAC TTG CGT GTT CTT GGA CTC CGT CCC AAG GAA GAG TAGA  
 Leu Glu his Leu Arg Val Leu Gly Leu Arg Pro Lys Glu Glu  
 1440 \*  
 AGAAAAAAG CGCCGAATGA AGTATTGCC CTTTTTCTC CAAGAATGGC AAAAGGAGAT  
 CAAGTGGACA TTCTCTATGA AGA

FIG. 3D

10 20 30 40 50 60<sup>\*</sup>  
LHHTY TNIAG ADPDVSTSEP DVRR IKPNQK W FVNHINQHM FV PFLYGLLA FKVR IQDINI  
70 80 90 100 110 120<sup>\*</sup>  
LYFVK TND A I RVNPISTWHT VMFWGGK AFF V WYRLI VPLQ YL PLGK VLLL FTVADMVSSY  
130 140 150 160 170 180<sup>\*</sup>  
WLA LTFQANY VVEEVQWPLP DENG I IQKD W AAMQVETTQD YAHDSLWTS ITGSLNYQXV

HHL FPH

FIG. 4

|          | 10           | 20            | 30              | 40           | 50           | 60         | 70 |
|----------|--------------|---------------|-----------------|--------------|--------------|------------|----|
| MA29     | MGTDOG-KTFTW | EEELAAHNTKDDL | LAIRGRVYDV      | TKFLSRHPGGVD | TLLGAGR      | DRVTPV     | 59 |
| MA524    | MAAAPSVRTFT  | RAEVLNAEALN   | EGKKDAEAP--FLMI | IDNKVYDV     | REFVPDHPGGSV | ILTHV-GKDG | 67 |
| BorD6    | MA-----      | AQIKKYITSD    | ELKNHDKPGDLWI   | SIQGKAYDV    | SDWVKDHPGGSF | PLKSLAGQEV | 59 |
| Sy6803D6 | ML-TAE-RI    | -----         | -----           | -----        | -----        | -----      | 7  |
| Sp1D6    | MTSTTS-KV    | -----         | -----           | -----        | -----        | -----      | 8  |

[illegible]

|          | 150  | 160 | 170 | 180 | 190 | 200 | 210 |
|----------|--|-----|-----|-----|-----|-----|-----|
| MA29     | SLIASY AQLFVPEFVVERTWLQVVFAIIMGFACAQVGLNPLHDAASHFSVTHNPTVWKILGATHDFFNGAS |     |     |     |     |     | 199 |
| MA524    | IWGL--STVIVAKWGQTSTLANVLSAALLGLFWQQCGW-LAHDFLHQV[FQDRFWGDLFGAF LGGVCCQGF |     |     |     |     |     | 200 |
| BorD6    | FIAMLFAMSVYGVLFCEGLVHLFSGCLMGFLWIQSGW-IGHDAGHYMVVSDSRLNKFMGIFAANCLSGI    |     |     |     |     |     | 187 |
| Sy6803D6 | WLFSAW--AFVLFIAPVIFPVRLLGCMVLAIALAASFNVGHDANHNAYSNPHINRVLGMTYDFVGLSS     |     |     |     |     |     | 116 |
| Sp1D6    | WVVS AW--TFVVFEGPDVLMKLLGCIVLFGFVSAVGFNISHDGNHGGYSKYQWVNYLSGLTHDAIGVSS   |     |     |     |     |     | 117 |

**FIG. 5A**

|          | 360                           | 370                                       | 380                             | 390 | 400 | 410 | 420 |
|----------|-------------------------------|---|---------------------------------|-----|-----|-----|-----|
| MA29     | ADMVSSYWLALTFQANHVVEEVQWPLPDE | NGI IQKDWAAMQVETTDQYAHDSHLWTSITGSLNYQAVHH |                                 |     |     |     | 391 |
| MA524    | SQAVCGNLLAIVFSLNHNGMPVI       | -----SKEEAVDMDFFTKQIITGRD                 | VHPG-LFANWFTGGLNYQIEHH          |     |     |     | 399 |
| BorD6    | SLSVTG-MQVQVQFSLNHFESSVY      | -----V-GKPKGNWFEKQTDGTLDI                 | SCP-PWMDWFHGGGLQFQIEHH          |     |     |     | 377 |
| Sy6803D6 | TYMTYGI VVCTIFMLAHVLESTEF     | LPDGESGAIDDEWAI                           | CQIRTTANFATNNPFWNWFCGGLNHQVTHH  |     |     |     | 307 |
| Sp1D6    | VYMTHGLVACVVFMLAHVIEPAEFLDP   | NL--HIDDEWAI                              | AQVKTTVDFAFPNNPIINWVVGGLNYQTVHH |     |     |     | 306 |

**FIG. 5B**

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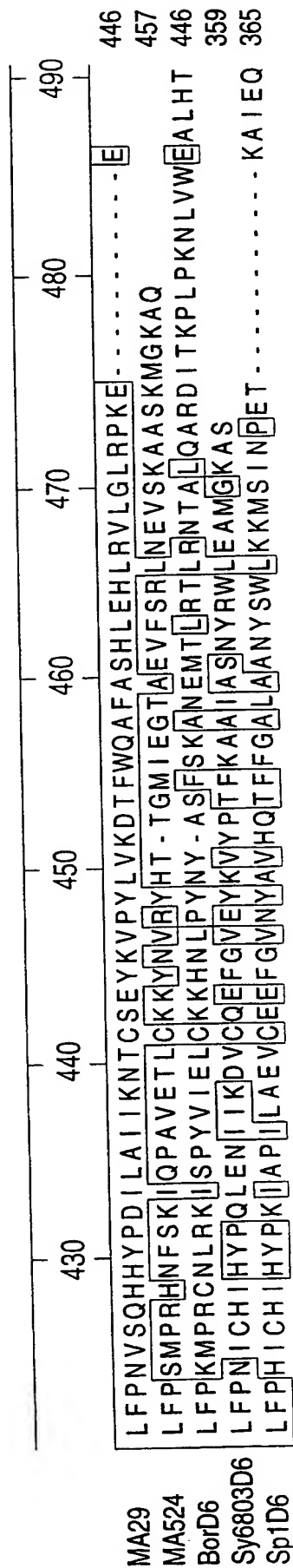


FIG. 5C

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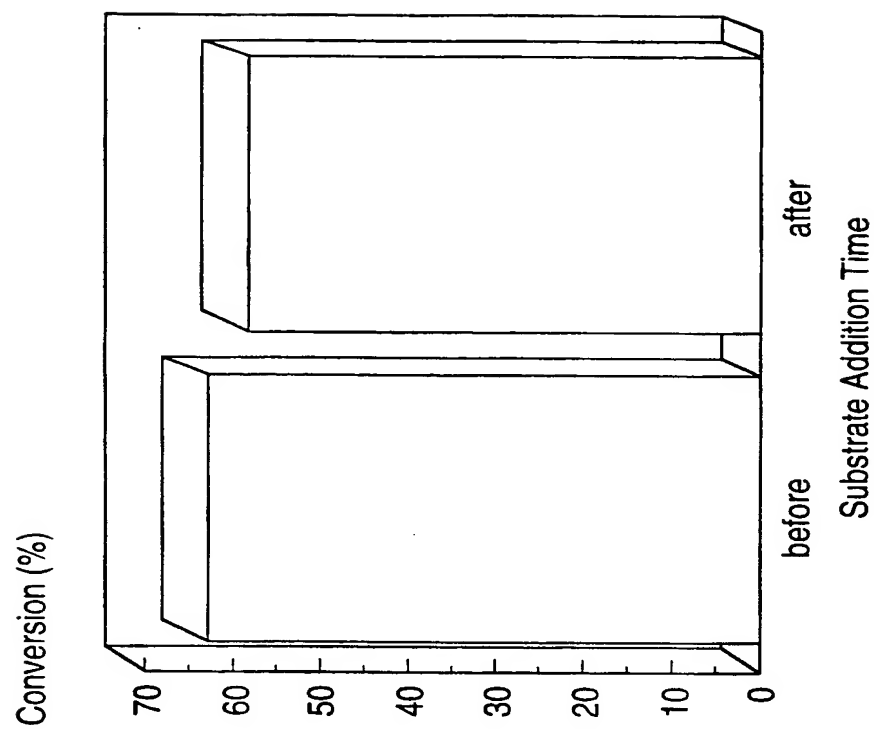


FIG. 6B

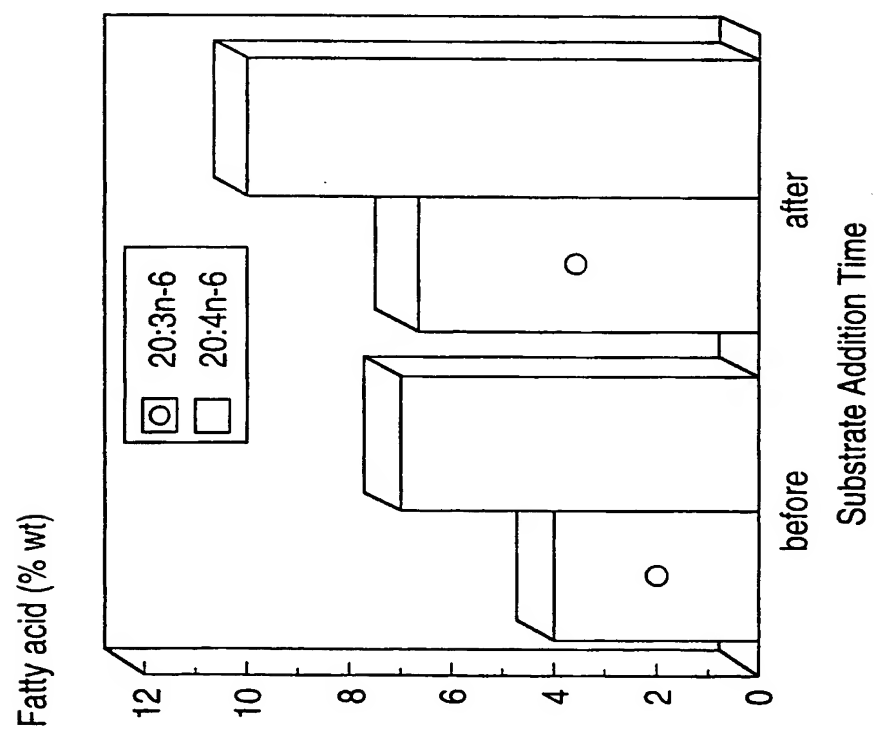


FIG. 6A

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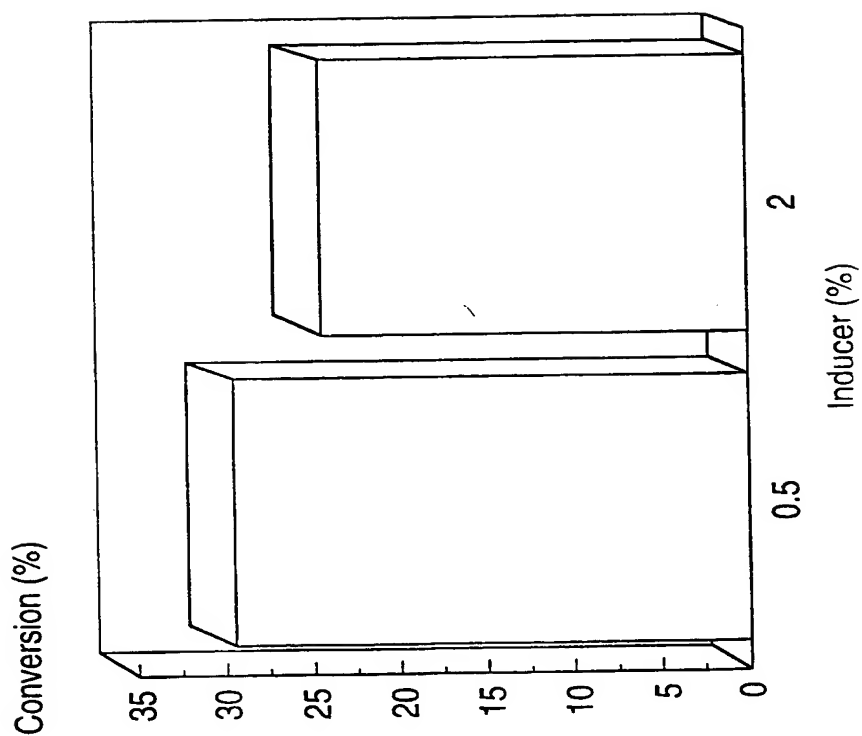


FIG. 7B

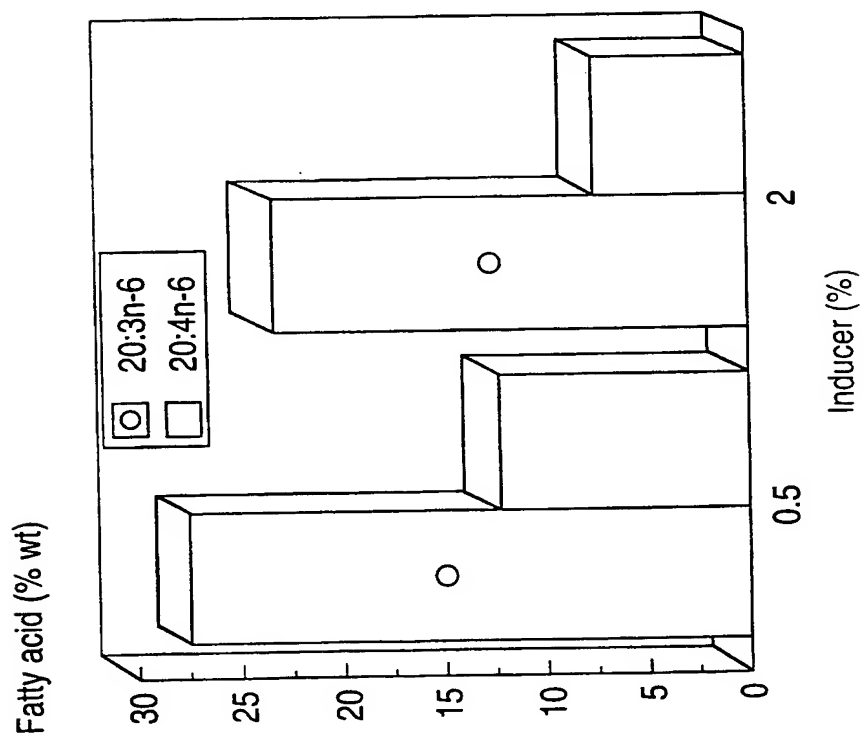


FIG. 7A

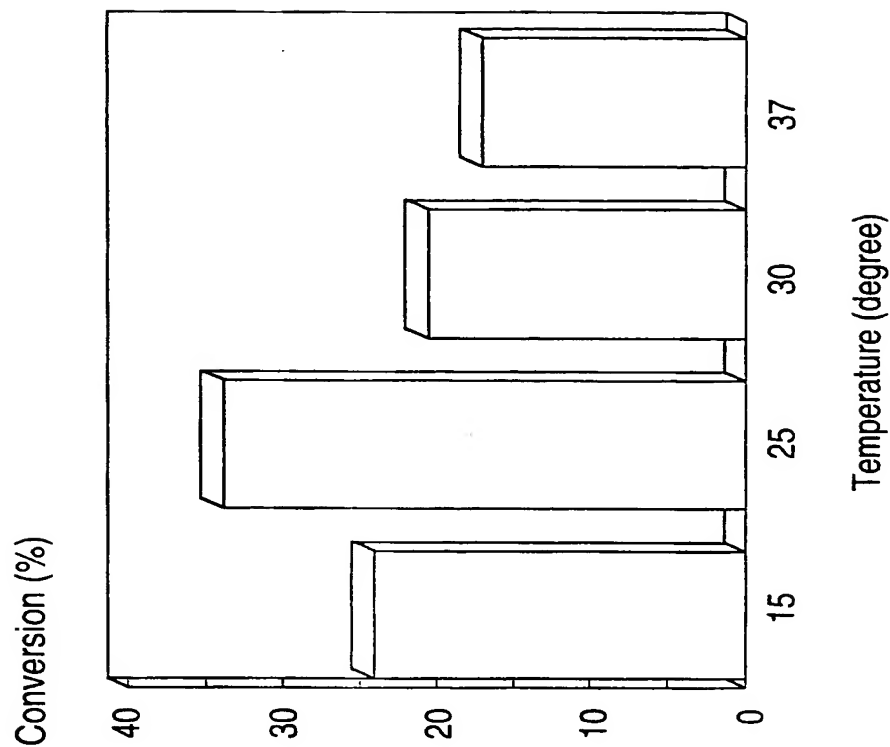


FIG. 8B

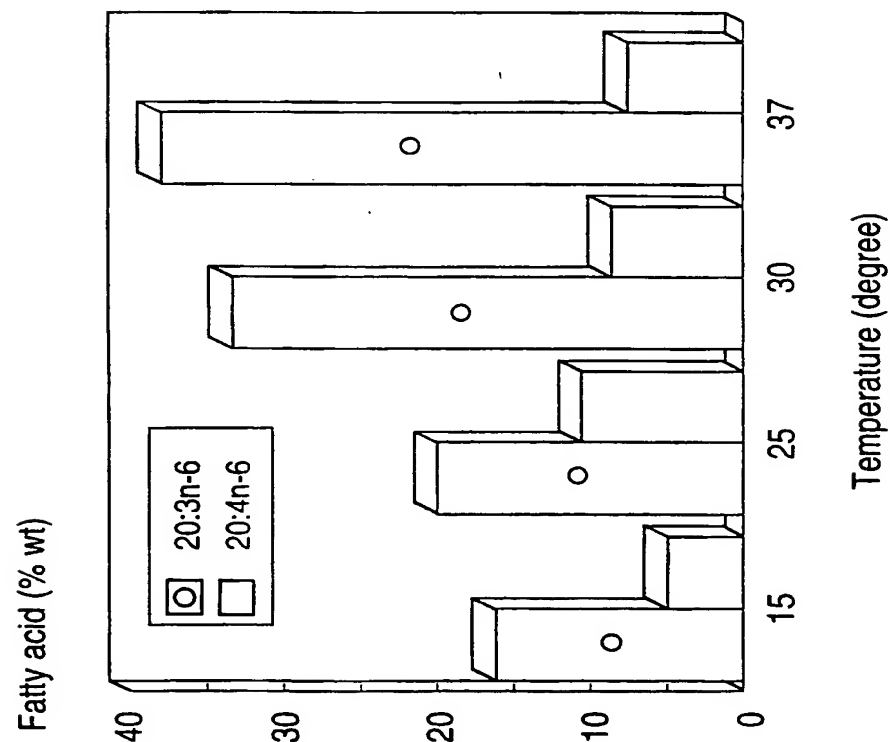
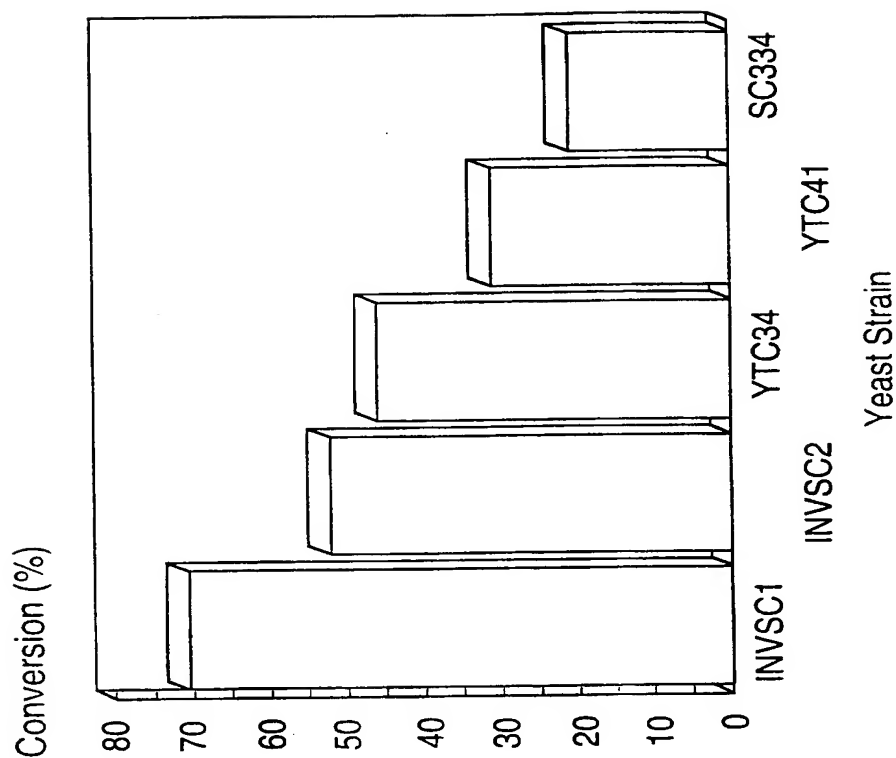


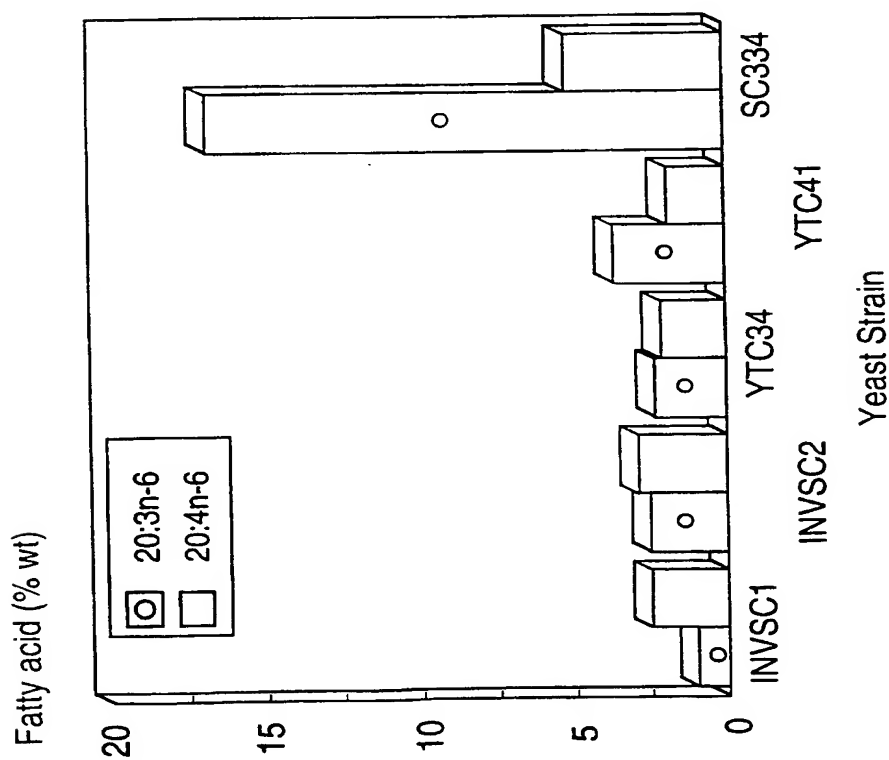
FIG. 8A



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**FIG. 9B**



**FIG. 9A**

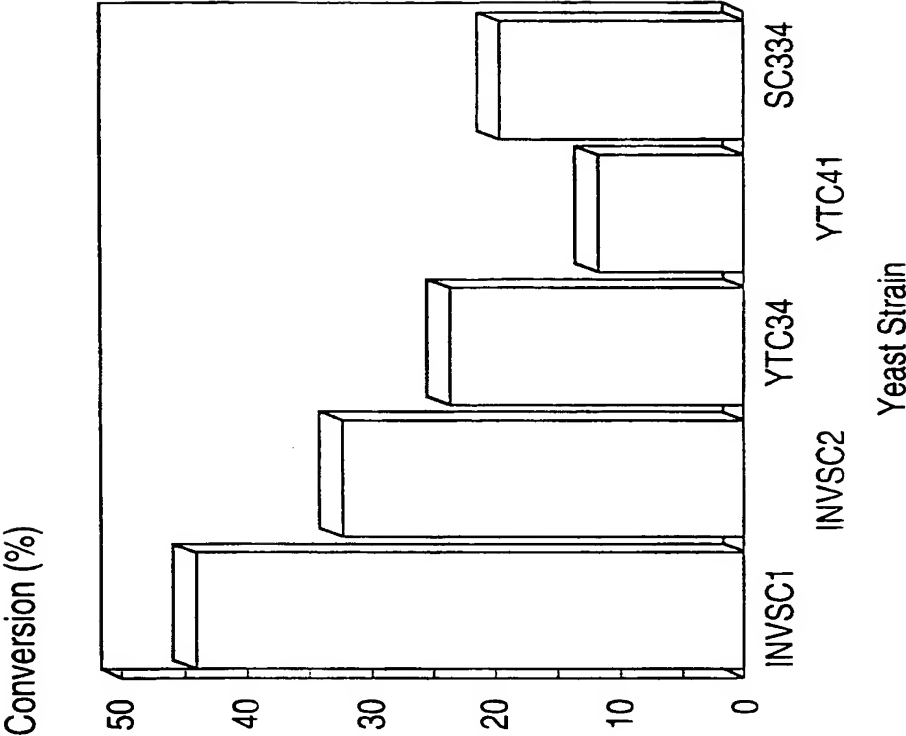


FIG. 10B

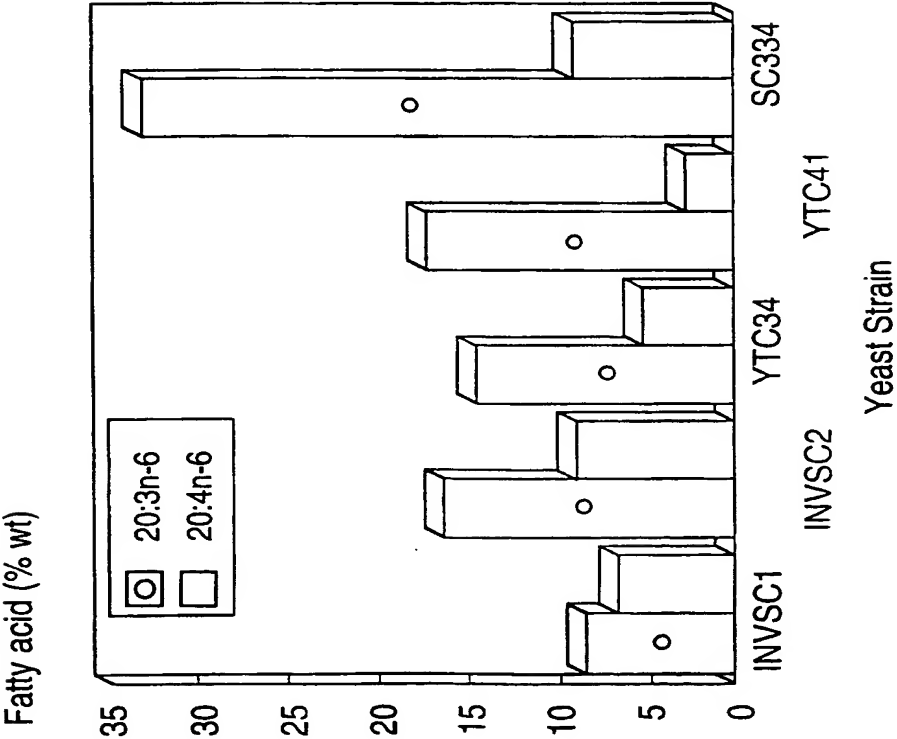


FIG. 10A

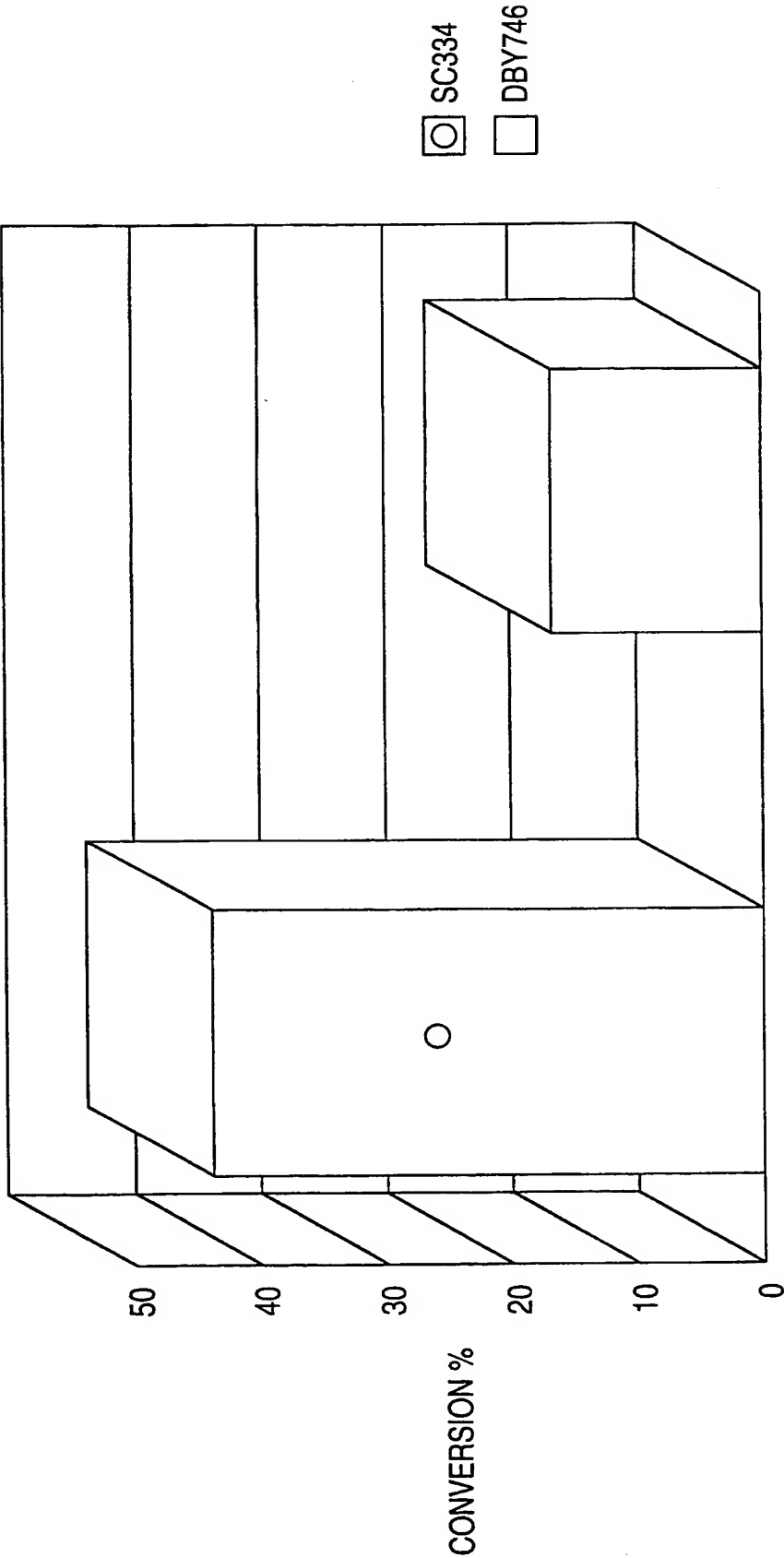


FIG. 11

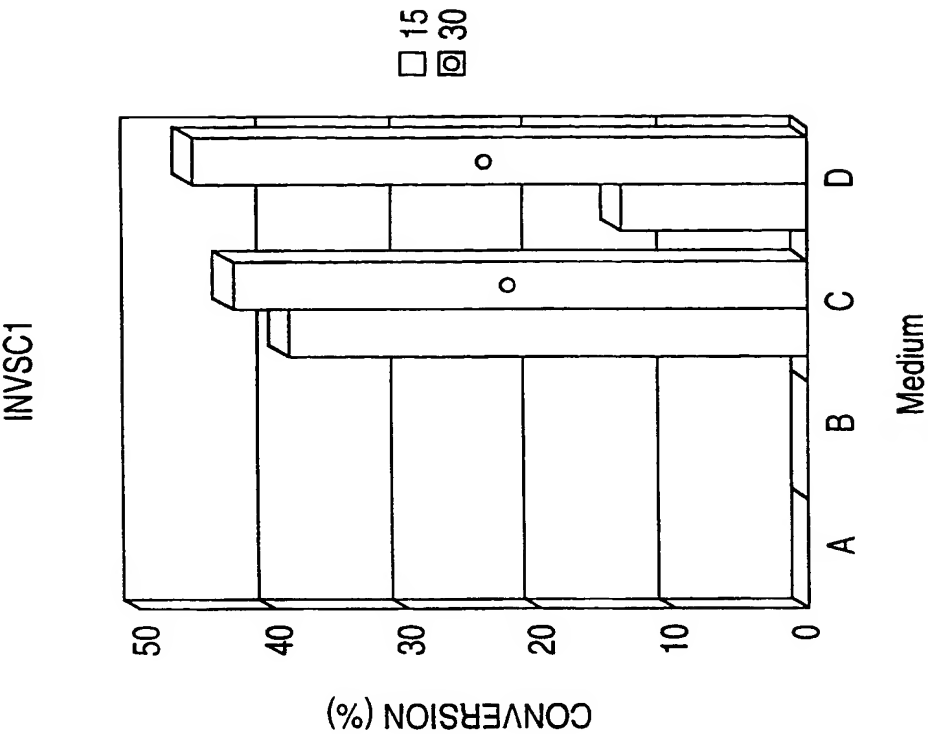


FIG. 12B

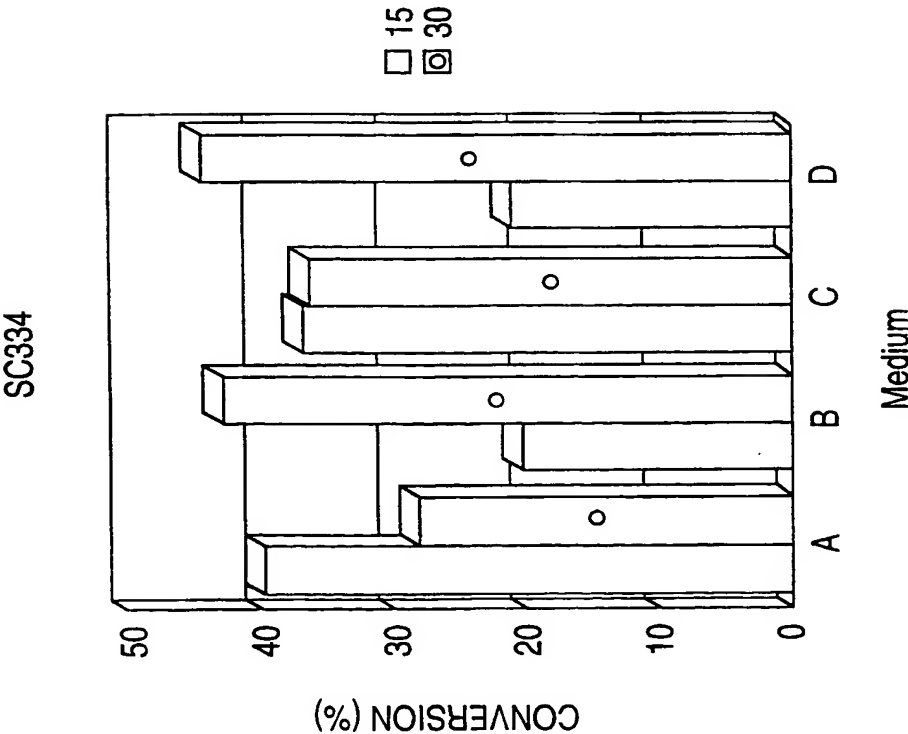


FIG. 12A

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SCORES INIT1: 117 INITN: 225 OPT: 256  
SMITH-WATERMAN SCORE: 408; 27.0% IDENTITY IN 441 aa OVERLAP

|             |   |                         |
|-------------|---|-------------------------|
| ma29gcg.pep | MGTDQGKT - - - FTWEELAAHNTKDDLLA IRGRVYDVTKFLSRHPGGVDTL LLGAGRDVT     | 10 20 30 40 50          |
| 253538a     | QGPTPRYFTWDEVAQRSGCEERWLVIDRKVYNISEFTRRHPGGSRVISHYAGQDAT              | 10 20 30 40 50          |
| ma29gcg.pep | PVFEMYHAF - GAADA IMKKYYVGTLSNELPIFPEPTVFHKT IKTRVEGYFTDRNIDPKN       | 60 70 80 90 100 110     |
| 253538a     | DPFVAFHINKGLVKKYMNSLLIGEL - SPEQPSF - EPTKNKELTDEFREL RATVERMGLMK     | 60 70 80 90 100 110     |
| ma29gcg.pep | RPEIWGRYALIFGSLIASYYAQLFVPFVVERTWLQVVF - A I IMGFACAQVGLNPLHDASH      | 120 130 140 150 160 170 |
| 253538a     | ANHVF - - FLLYLLHILLDGAAWLT LWVFGTSFLPFLLCVALLSAVQAQAGWLQ - HDYGH     | 120 130 140 150 160 170 |
| ma29gcg.pep | FSVTHNPTVWKILGATHDF - - - FNGASYLVVMYQHMLGHHPTYNIAGADPDVSTSE - - -    | 180 190 200 210 220     |
| 253538a     | LSVYRKPK - WNHL - - VHKFVIGHLKGASANWNNHRRH - FQHHAKPNI FHKDPDVNMLHVFV | 180 190 200 210 220     |

FIG. 13A

**FIG. 13B**

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SCORES INIT1: 231 INITN: 499 OPT: 401  
SMITH-WATERMAN SCORE: 620; 27.3% IDENTITY IN 455 aa OVERLAP

|              |   |  |     |
|--------------|---|--|-----|
| ma524gcg.pep | MAAAPSVRTFTTAEVLNAEALNEGKKDAEAPFLMI     | IDNKVYDVREFVDPDHPGGSVILTH-                   | 59  |
|              | :                                       | :  | 50  |
| 253538a      | QGPTPRYFTWDEV                           | -----AQRSGCEERWLVIDRKVYNI SEFTRRHPPGGSRVISHY | 50  |
|              | 10                                      | 20   | 30  |
| ma524gcg.pep | VGKDGTDVFDTFHPEAAW--ETLANFYVGDIDE---    | SDRDIKNDDFAAEVRKLRTLFQSL                     | 110 |
|              | :                                       | :  | 100 |
| 253538a      | AGQDATDPFVAFHINKGLVKKYMNLSLIGELSP       | EQSPSEPTKNKELTDEFREL RATVERM                 | 110 |
|              | 60                                      | 70   | 80  |
| ma524gcg.pep | GYDSSKAYYAFKVSFNLCIWGLSTVI              | VAKWGQTSTLANVLSAALLGLFWQQCGWLAHDF            | 170 |
|              | : : : : : : : :   :   :   :   :   :   : | :   :   :   :   :   :   :   :   :   :        | 160 |
| 253538a      | GLMKANHVVFFLLYLLHILLDGAAWLTLWVFG-       | TSFLPFLLCAVLLSAVQAQAGWLQHDY                  | 160 |
|              | 120                                     | 130  | 140 |
| ma524gcg.pep | LHHQVFOQDRFWGDLFGAFLGGVCQGFSSW          | WKDKHNTHHAAPNVHGEDPDIDTHPLLTWS               | 230 |
|              | :   : :   :   :   :   :   :   :   :   : | :  | 220 |
| 253538a      | GHL SVYRKPKWNHLVHKFVIGHLKGASANW         | NHRHFQHHAKPNI FHKDPDVN-----ML---             | 220 |
|              | 170                                     | 180  | 190 |

FIG. 14A

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SCORES INIT1: 231 INITN: 499 OPT: 401  
SMITH-WATERMAN SCORE: 620; 27.3% IDENTITY IN 455 aa OVERLAP

|              |  |     |     |     |     |     |
|--------------|--|-----|-----|-----|-----|-----|
| ma524gcg.pep | 240  | 250 | 260 | 270 | 280 | 290 |
|              | EHALEMFSVPDEELTRMWSRFMVLNQTWYFPILS--FARLSWCLQSILFVLPNGQAH      |     |     |     |     |     |
|              | :::  | ::: | ::: | ::: | ::: | ::: |
| 253538a      | -HVF-VLGEWQPIEYGKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQIMTMI-----VH    | 230 | 240 | 250 | 260 | 270 |
| ma524gcg.pep | 300  | 310 | 320 | 330 | 340 | 349 |
|              | KPSGARVPISLVEQLSLAMHWTWYLATMFLFIK--DPVNMLVYFLVSQAVCGNLLAIVFS   |     |     |     |     |     |
|              | :::  | ::: | ::: | ::: | ::: | ::: |
| 253538a      | K-----NWVDLAWAVSYIRFFITYIPFYGILGALLFLNFI RFLESHWFWVWTQ         | 280 | 290 | 300 | 310 | 320 |
| ma524gcg.pep | 350  | 360 | 370 | 380 | 390 | 400 |
|              | LNHNMGMPVISKEEAVDMDFFTKQIITGRDVHPGLFANWFTGGLNYQIEHHLFPSMPRHNF  |     |     |     |     |     |
|              | :::  | ::: | ::: | ::: | ::: | ::: |
| 253538a      | MNHI VMEI--DQEAYR-DWFFSSQLTATCNVEQSFFNDWFSGHLNFQIEHHLFPTMPRHNL | 330 | 340 | 350 | 360 | 370 |
| ma524gcg.pep | 410  | 420 | 430 | 440 | 450 |     |
|              | SKIQPAVETLCKKYNVRYHTTGMIETAEVFSRLNEVSKAASKMGKAQX               |     |     |     |     |     |
|              | :::  | ::: | ::: | ::: | ::: | ::: |
| 253538a      | HKIAPLVKSLCAKHGIEYQEKPLLRALLDIRSLKKGKGLWLDAYLHKX               | 390 | 400 | 410 | 420 | 430 |

FIG. 14B



## SEQUENCE LISTING

5 <110> Mukerji, Pradip  
Huang, Yung-Sheng  
Parker-Barnes, Jennifer M.  
Das, Tapas

10 <120> DELTA FIVE DESATURASES AND ALTERED FATTY ACID  
BIOSYNTHESIS AND PRODUCTS PRODUCED THEREFROM

<130> CGAB-400A

15 <140>  
<141>

<150> U.S. 60/103,109  
<151> 1998-10-05

20 <160> 38

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25 <212> DNA  
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cgacctactc ttggccatcc gcggcagggt gtacgatgtc acaaagttct tgagccgcca 180  
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gatgtatcac gcgtttgggg ctgcagatgc cattatgaag aagtactatg tcggtacact 300  
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gagagtcgag ggctacttta cggatcggaa cattgatccc aagaatagac cagagatctg 420  
gggacgatac gctcttatct ttggatcctt gatcgcttcc tactacgcgc agctctttgt 480  
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gtgcgacaaa gtgggactca accctcttca tgatgcgtct cacttttcag tgacccacaa 600  
50 cccactgtc tggaagattc tgggagccac gcacgacttt ttcaacggag catcgtaact 660  
ggtgtggatg taccaacata tgctcggcca tcaccctac accaacattg ctggagcaga 720  
tcccgacgtg tcgacgtctg agcccgatgt tcgtcgtatc aagcccaacc aaaagtgggt 780  
55 tgtcaaccac atcaaccagc acatgtttgt tcttttctg tacggactgc tggcgttcaa 840  
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60 caateccatc tcgacatggc aactgtgat gttctggggc ggcaaggctt tctttgtctg 960  
gtatcgcctg attgttcccc tgcagtatct gcccttgggc aaggtgctgc tcttgttcac 1020  
ggtcgcggaac atggtgtcgt cttactggct ggcgctgacc ttccaggcga accacgttgt 1080  
65 tgaggaagtt cagtggccgt tgctgacga gaacgggac atccaaaagg actgggcagc 1140  
tatgcaggtc gagactacgc aggattacgc acacgattcg cacctctgga ccagcatcac 1200

tggcagcttg aactaccagg ctgtgcacca tctgttcccc aacgtgtcgc agcaccatta 1260  
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 5 caaggatacg ttttggcaag catttgcttc acatttggag cacttgcgtg ttcttggact 1380  
 ccgtcccaag gaagagtaga agaaaaaag cgccgaatga agtattgccc cctttttctc 1440  
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 20 Met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Glu Leu Ala Ala  
 1 5 10 15  
 His Asn Thr Lys Asp Asp Leu Leu Leu Ala Ile Arg Gly Arg Val Tyr  
 20 25 30  
 25 Asp Val Thr Lys Phe Leu Ser Arg His Pro Gly Gly Val Asp Thr Leu  
 35 40 45  
 Leu Leu Gly Ala Gly Arg Asp Val Thr Pro Val Phe Glu Met Tyr His  
 50 55 60  
 30 Ala Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr  
 65 70 75 80  
 35 Leu Val Ser Asn Glu Leu Pro Ile Phe Pro Glu Pro Thr Val Phe His  
 85 90 95  
 Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Thr Asp Arg Asn Ile  
 100 105 110  
 40 Asp Pro Lys Asn Arg Pro Glu Ile Trp Gly Arg Tyr Ala Leu Ile Phe  
 115 120 125  
 Gly Ser Leu Ile Ala Ser Tyr Tyr Ala Gln Leu Phe Val Pro Phe Val  
 130 135 140  
 45 Val Glu Arg Thr Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe  
 145 150 155 160  
 50 Ala Cys Ala Gln Val Gly Leu Asn Pro Leu His Asp Ala Ser His Phe  
 165 170 175  
 Ser Val Thr His Asn Pro Thr Val Trp Lys Ile Leu Gly Ala Thr His  
 180 185 190  
 55 Asp Phe Phe Asn Gly Ala Ser Tyr Leu Val Trp Met Tyr Gln His Met  
 195 200 205  
 Leu Gly His His Pro Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val  
 210 215 220  
 60 Ser Thr Ser Glu Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp  
 225 230 235 240  
 65 Phe Val Asn His Ile Asn Gln His Met Phe Val Pro Phe Leu Tyr Gly  
 245 250 255  
 Leu Leu Ala Phe Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe  
 260 265 270  
 70 Val Lys Thr Asn Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His

|     | 275  | 280  | 285 |
|-----|--|--|-----|
| 5   | Thr Val Met Phe Trp Gly Gly<br>290 295   | Lys Ala Phe Phe Val Trp Tyr Arg Leu<br>300 |     |
| 10  | Ile Val Pro Leu Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Leu Phe<br>305 310 315 320 |  |     |
| 15  | Thr Val Ala Asp Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln<br>325 330 335     |  |     |
| 20  | Ala Asn His Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn<br>340 345 350     |  |     |
| 25  | Gly Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln<br>355 360 365     |  |     |
| 30  | Asp Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu<br>370 375 380     |  |     |
| 35  | Asn Tyr Gln Ala Val His His Leu Phe Pro Asn Val Ser Gln His His<br>385 390 400     |  |     |
| 40  | Tyr Pro Asp Ile Leu Ala Ile Ile Lys Asn Thr Cys Ser Glu Tyr Lys<br>405 410 415     |  |     |
| 45  | Val Pro Tyr Leu Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His<br>420 425 430     |  |     |
| 50  | Leu Glu His Leu Arg Val Leu Gly Leu Arg Pro Lys Glu Glu<br>435 440 445             |  |     |
| 55  | <210> 3<br><211> 186<br><212> PRT<br><213> Mortierella alpina                      |  |     |
| 60  | <400> 3  |  |     |
| 65  | Leu His His Thr Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val Ser<br>1 5 10 15       |  |     |
| 70  | Thr Ser Glu Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp Phe<br>20 25 30        |  |     |
| 75  | Val Asn His Ile Asn Gln His Met Phe Val Pro Phe Leu Tyr Gly Leu<br>35 40 45        |  |     |
| 80  | Leu Ala Phe Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe Val<br>50 55 60        |  |     |
| 85  | Lys Thr Asn Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His Thr<br>65 70 75 80     |  |     |
| 90  | Val Met Phe Trp Gly Gly Lys Ala Phe Phe Val Trp Tyr Arg Leu Ile<br>85 90 95        |  |     |
| 95  | Val Pro Leu Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Leu Phe Thr<br>100 105 110     |  |     |
| 100 | Val Ala Asp Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln Ala<br>115 120 125     |  |     |
| 105 | Asn Tyr Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn Gly<br>130 135 140     |  |     |
| 110 | Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln Asp<br>145 150 155 160 |  |     |

Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu Asn  
                                   165                                  170                                  175  
 5 Tyr Gln Xaa Val His His Leu Phe Pro His  
                                   180                                  185  
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     1                                  5                                  10                                  15  
     Asn Ala Glu Ala Leu Asn Glu Gly Lys Lys Asp Ala Glu Ala Pro Phe  
                                   20                                  25                                  30  
 20 Leu Met Ile Ile Asp Asn Lys Val Tyr Asp Val Arg Glu Phe Val Pro  
                                   35                                  40                                  45  
 25 Asp His Pro Gly Gly Ser Val Ile Leu Thr His Val Gly Lys Asp Gly  
     50                                  55                                  60  
     Thr Asp Val Phe Asp Thr Phe His Pro Glu Ala Ala Trp Glu Thr Leu  
     65                                  70                                  75                                  80  
 30 Ala Asn Phe Tyr Val Gly Asp Ile Asp Glu Ser Asp Arg Asp Ile Lys  
                                   85                                  90                                  95  
     Asn Asp Asp Phe Ala Ala Glu Val Arg Lys Leu Arg Thr Leu Phe Gln  
                                   100                                  105                                  110  
 35 Ser Leu Gly Tyr Tyr Asp Ser Ser Lys Ala Tyr Tyr Ala Phe Lys Val  
                                   115                                  120                                  125  
 40 Ser Phe Asn Leu Cys Ile Trp Gly Leu Ser Thr Val Ile Val Ala Lys  
     130                                  135                                  140  
     Trp Gly Gln Thr Ser Thr Leu Ala Asn Val Leu Ser Ala Ala Leu Leu  
     145                                  150                                  155                                  160  
 45 Gly Leu Phe Trp Gln Gln Cys Gly Trp Leu Ala His Asp Phe Leu His  
                                   165                                  170                                  175  
     His Gln Val Phe Gln Asp Arg Phe Trp Gly Asp Leu Phe Gly Ala Phe  
                                   180                                  185                                  190  
 50 Leu Gly Gly Val Cys Gln Gly Phe Ser Ser Ser Trp Trp Lys Asp Lys  
                                   195                                  200                                  205  
 55 His Asn Thr His His Ala Ala Pro Asn Val His Val Glu Asp Pro Asp  
     210                                  215                                  220  
     Ile Asp Thr His Pro Leu Leu Thr Trp Ser Glu His Ala Leu Glu Met  
     225                                  230                                  235                                  240  
 60 Phe Ser Asp Val Pro Asp Glu Glu Leu Thr Arg Met Trp Ser Arg Phe  
                                   245                                  250                                  255  
     Met Val Leu Asn Gln Thr Trp Phe Tyr Phe Pro Ile Leu Ser Phe Ala  
                                   260                                  265                                  270  
 65 Arg Leu Ser Trp Cys Leu Gln Ser Ile Leu Phe Val Leu Pro Asn Gly  
                                   275                                  280                                  285  
 70 Gln Ala His Lys Pro Ser Gly Ala Arg Val Pro Ile Ser Leu Val Glu  
     290                                  295                                  300

Gln Leu Ser Leu Ala Met His Trp Thr Trp Tyr Leu Ala Thr Met Phe  
 305 310 315 320  
 5 Leu Phe Ile Lys Asp Pro Val Asn Met Leu Val Tyr Phe Leu Val Ser  
 325 330 335  
 Gln Ala Val Cys Gly Asn Leu Leu Ala Ile Val Phe Ser Leu Asn His  
 340 345 350  
 10 Asn Gly Met Pro Val Ile Ser Lys Glu Glu Ala Val Asp Met Asp Phe  
 355 360 365  
 15 Phe Thr Lys Gln Ile Ile Thr Gly Arg Asp Val His Pro Gly Leu Phe  
 370 375 380  
 Ala Asn Trp Phe Thr Gly Gly Leu Asn Tyr Gln Ile Glu His His Leu  
 385 390 395 400  
 20 Phe Pro Ser Met Pro Arg His Asn Phe Ser Lys Ile Gln Pro Ala Val  
 405 410 415  
 Glu Thr Leu Cys Lys Lys Tyr Asn Val Arg Tyr His Thr Thr Gly Met  
 420 425 430  
 25 Ile Glu Gly Thr Ala Glu Val Phe Ser Arg Leu Asn Glu Val Ser Lys  
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 30 Ala Ala Ser Lys Met Gly Lys Ala Gln  
 450 455  
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 1 5 10 15  
 His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr  
 20 25 30  
 45 Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu  
 35 40 45  
 50 Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His  
 50 55 60  
 Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr  
 65 70 75 80  
 55 Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Val Tyr Arg Lys Leu  
 85 90 95  
 Val Phe Glu Phe Ser Lys Met Gly Leu Tyr Asp Lys Lys Gly His Ile  
 100 105 110  
 60 Met Phe Ala Thr Leu Cys Phe Ile Ala Met Leu Phe Ala Met Ser Val  
 115 120 125  
 Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Phe Ser Gly  
 130 135 140  
 65 Cys Leu Met Gly Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp  
 145 150 155 160  
 70 Ala Gly His Tyr Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met

|    | 165                 | 170                         | 175  |
|----|---------------------|-----------------------------|--|
| 5  | Gly Ile Phe Ala 180 | Ala Asn Cys Leu Ser 185     | Gly Ile Ser Ile Gly Trp Trp 190            |
| 10 | Lys Trp Asn 195     | His Asn Ala His 200         | Ile Ala Cys Asn Ser 205                    |
| 15 | Asp Pro Asp 210     | Leu Gln Tyr Ile 215         | Pro Phe Leu Val Val Ser Ser Lys Phe 220    |
| 20 | Phe Gly Ser 225     | Leu Thr Ser His 230         | Phe Tyr Glu Lys Arg Leu Thr Phe Asp 240    |
| 25 | Ser Leu Ser 245     | Arg Phe Phe Val Ser Tyr 250 | Gln His Trp Thr Phe Tyr Pro 255            |
| 30 | Ile Met Cys 260     | Ala Ala Arg Leu Asn 265     | Met Tyr Val Gln Ser Leu Ile Met 270        |
| 35 | Leu Leu Thr 275     | Lys Arg Asn Val Ser Tyr 280 | Arg Ala Gln Glu Leu Leu Gly 285            |
| 40 | Cys Leu Val 290     | Phe Ser Ile Trp Tyr 295     | Pro Leu Leu Val Ser Cys Leu Pro 300        |
| 45 | Asn Trp Gly 305     | Glu Arg Ile Met Phe Val 310 | Ile Ala Ser Leu Ser Val Thr 315 320        |
| 50 | Gly Met Gln 325     | Gln Phe Ser Leu Asn 330     | His Phe Ser Ser Ser Val 335                |
| 55 | Tyr Val Gly 340     | Lys Pro Lys Gly Asn 345     | Asn Trp Phe Glu Lys Gln Thr Asp 350        |
| 60 | Gly Thr Leu 355     | Asp Ile Ser Cys Pro 360     | Pro Trp Met Asp Trp Phe His Gly 365        |
| 65 | Gly Leu Gln 370     | Phe Gln Ile Glu His 375     | His Leu Phe Pro Lys Met Pro Arg 380        |
| 70 | Cys Asn Leu 385     | Arg Lys Ile Ser Pro Tyr 390 | Val Ile Glu Leu Cys Lys Lys 395 400        |
|    | His Asn Leu 405     | Pro Tyr Asn Tyr Ala Ser 410 | Phe Ser Lys Ala Asn Glu Met 415            |
|    | Thr Leu Arg 420     | Thr Leu Arg Asn Thr 425     | Ala Leu Gln Ala Arg Asp Ile Thr 430        |
|    | Lys Pro Leu 435     | Pro Lys Asn Leu Val 440     | Trp Glu Ala Leu His Thr 445                |
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| 70 | Thr Gln Arg 35      | Asp Asn Pro Ser Met 40      | Tyr Leu Lys Thr Leu Ile Ile Val 45         |

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 15 Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly  
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 165 170 175  
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|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|    | Arg | Lys | Glu | Leu | Asn | Arg | Arg | Val | Asn | Ala | Tyr | Leu | Glu | Ala | Glu | Asn |  |
|    |     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |  |
| 5  | Ile | Ser | Pro | Arg | Asp | Asn | Pro | Pro | Met | Tyr | Leu | Lys | Thr | Ala | Ile | Ile |  |
|    |     |     | 35  |     |     |     |     | 40  |     |     |     | 45  |     |     |     |     |  |
|    | Leu | Ala | Trp | Val | Val | Ser | Ala | Trp | Thr | Phe | Val | Val | Phe | Gly | Pro | Asp |  |
| 10 |     | 50  |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     |  |
|    | Val | Leu | Trp | Met | Lys | Leu | Leu | Gly | Cys | Ile | Val | Leu | Gly | Phe | Gly | Val |  |
|    | 65  |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     |     | 80  |  |
| 15 | Ser | Ala | Val | Gly | Phe | Asn | Ile | Ser | His | Asp | Gly | Asn | His | Gly | Gly | Tyr |  |
|    |     |     |     |     | 85  |     |     |     |     | 90  |     |     |     |     | 95  |     |  |
|    | Ser | Lys | Tyr | Gln | Trp | Val | Asn | Tyr | Leu | Ser | Gly | Leu | Thr | His | Asp | Ala |  |
|    |     |     |     | 100 |     |     |     |     | 105 |     |     |     |     | 110 |     |     |  |
| 20 | Ile | Gly | Val | Ser | Ser | Tyr | Leu | Trp | Lys | Phe | Arg | His | Asn | Val | Leu | His |  |
|    |     |     | 115 |     |     |     |     | 120 |     |     |     |     | 125 |     |     |     |  |
|    | His | Thr | Tyr | Thr | Asn | Ile | Leu | Gly | His | Asp | Val | Glu | Ile | His | Gly | Asp |  |
| 25 |     |     | 130 |     |     |     | 135 |     |     |     |     | 140 |     |     |     |     |  |
|    | Glu | Leu | Val | Arg | Met | Ser | Pro | Ser | Met | Glu | Tyr | Arg | Trp | Tyr | His | Arg |  |
|    | 145 |     |     |     |     | 150 |     |     |     |     | 155 |     |     |     |     | 160 |  |
| 30 | Tyr | Gln | His | Trp | Phe | Ile | Trp | Phe | Val | Tyr | Pro | Phe | Ile | Pro | Tyr | Tyr |  |
|    |     |     |     |     | 165 |     |     |     |     | 170 |     |     |     |     | 175 |     |  |
|    | Trp | Ser | Ile | Ala | Asp | Val | Gln | Thr | Met | Leu | Phe | Lys | Arg | Gln | Tyr | His |  |
|    |     |     |     | 180 |     |     |     |     | 185 |     |     |     |     | 190 |     |     |  |
| 35 | Asp | His | Glu | Ile | Pro | Ser | Pro | Thr | Trp | Val | Asp | Ile | Ala | Thr | Leu | Leu |  |
|    |     |     | 195 |     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |  |
|    | Ala | Phe | Lys | Ala | Phe | Gly | Val | Ala | Val | Phe | Leu | Ile | Ile | Pro | Ile | Ala |  |
| 40 |     |     | 210 |     |     |     | 215 |     |     |     |     | 220 |     |     |     |     |  |
|    | Val | Gly | Tyr | Ser | Pro | Leu | Glu | Ala | Val | Ile | Gly | Ala | Ser | Ile | Val | Tyr |  |
|    | 225 |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     |     | 240 |  |
| 45 | Met | Thr | His | Gly | Leu | Val | Ala | Cys | Val | Val | Phe | Met | Leu | Ala | His | Val |  |
|    |     |     |     |     | 245 |     |     |     |     | 250 |     |     |     |     | 255 |     |  |
|    | Ile | Glu | Pro | Ala | Glu | Phe | Leu | Asp | Pro | Asp | Asn | Leu | His | Ile | Asp | Asp |  |
|    |     |     |     | 260 |     |     |     |     | 265 |     |     |     |     | 270 |     |     |  |
| 50 | Glu | Trp | Ala | Ile | Ala | Gln | Val | Lys | Thr | Thr | Val | Asp | Phe | Ala | Pro | Asn |  |
|    |     |     | 275 |     |     |     |     | 280 |     |     |     |     | 285 |     |     |     |  |
|    | Asn | Thr | Ile | Ile | Asn | Trp | Tyr | Val | Gly | Gly | Leu | Asn | Tyr | Gln | Thr | Val |  |
| 55 |     |     |     |     |     |     | 295 |     |     |     |     | 300 |     |     |     |     |  |
|    | His | His | Leu | Phe | Pro | His | Ile | Cys | His | Ile | His | Tyr | Pro | Lys | Ile | Ala |  |
|    | 305 |     |     |     |     | 310 |     |     |     |     | 315 |     |     |     |     | 320 |  |
| 60 | Pro | Ile | Leu | Ala | Glu | Val | Cys | Glu | Glu | Phe | Gly | Val | Asn | Tyr | Ala | Val |  |
|    |     |     |     |     | 325 |     |     |     |     | 330 |     |     |     |     | 335 |     |  |
|    | His | Gln | Thr | Phe | Phe | Gly | Ala | Leu | Ala | Ala | Asn | Tyr | Ser | Trp | Leu | Lys |  |
|    |     |     |     | 340 |     |     |     |     | 345 |     |     |     |     | 350 |     |     |  |
| 65 | Lys | Met | Ser | Ile | Asn | Pro | Glu | Thr | Lys | Ala | Ile | Glu | Gln |     |     |     |  |
|    |     |     | 355 |     |     |     |     | 360 |     |     |     |     | 365 |     |     |     |  |



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|    |       | aggatggtaa aaatgggtgca attcgtgtta gtgtcgccac aaatttcgat aaggccgctt 180  |  |
|    |       | acgtcattgg taaattgtct tttgttttct tccgtttcat ccttccactc cgttatcata 240   |  |
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|    |       | caattaattt ccaagttagt catgtcgtg aagatctcaa attcctttgct acccctgaaa 360   |  |
| 15 |       | gaccagatga accatctcaa atcaatgaag attgggcaat ccttcaactt aaaactactc 420   |  |
|    |       | aagattatgg tcatggttca ctcttttgta ccttttttag tggttcttta aatcatcaag 480   |  |
|    |       | ttgttcatca tttattccca tcaattgctc aagatttcta cccacaactt gtaccaattg 540   |  |
|    |       | taaaagaagt ttgtaaagaa cataacatta cttaccacat taaaccaaac ttcactgaag 600   |  |
|    |       | ctattatgtc acacattaat tacctttaca aaatgggtaa tgatccagat tatgttaaaa 660   |  |
| 20 |       | aaccattagc ctcaaaagat gattaaatga aataacttaa aaaccaatta tttacttttg 720   |  |
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| 35 |       | Arg Thr Gln Asp Trp Glu Ala Phe Val Lys Asp Gly Lys Asn Gly             |  |
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|    |       | Ala Ile Arg Val Ser Val Ala Thr Asn Phe Asp Lys Ala Ala Tyr             |  |
|    |       | 50 55 60  |  |
|    |       | Val Ile Gly Lys Leu Ser Phe Val Phe Phe Arg Phe Ile Leu Pro             |  |
|    |       | 65 70 75  |  |
| 40 |       | Leu Arg Tyr His Ser Phe Thr Asp Leu Ile Cys Tyr Phe Leu Ile             |  |
|    |       | 80 85 90  |  |
|    |       | Ala Glu Phe Val Phe Gly Trp Tyr Leu Thr Ile Asn Phe Gln Val             |  |
|    |       | 95 100 105  |  |
| 45 |       | Ser His Val Ala Glu Asp Leu Lys Phe Phe Ala Thr Pro Glu Arg             |  |
|    |       | 110 115 120   |  |
|    |       | Pro Asp Glu Pro Ser Gln Ile Asn Glu Asp Trp Ala Ile Leu Gln             |  |
|    |       | 125 130 135   |  |
|    |       | Leu Lys Thr Thr Gln Asp Tyr Gly His Gly Ser Leu Leu Cys Thr             |  |
|    |       | 140 145 150   |  |
| 50 |       | Phe Phe Ser Gly Ser Leu Asn His Gln Val Val His His Leu Phe             |  |
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|    |       | Pro Ser Ile Ala Gln Asp Phe Tyr Pro Gln Leu Val Pro Ile Val             |  |
|    |       | 170 175 180   |  |
| 55 |       | Lys Glu Val Cys Lys Glu His Asn Ile Thr Tyr His Ile Lys Pro             |  |
|    |       | 185 190 195   |  |
|    |       | Asn Phe Thr Glu Ala Ile Met Ser His Ile Asn Tyr Leu Tyr Lys             |  |
|    |       | 200 205 210   |  |
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|    | tgcaaggagt ggggtgtcca gtaccacgaa gccgacctcg tggacgggac catggaagtc    | 240 |
|    | ttgcaccatt tgggcagcgt ggccggcgaa ttcgtcgtgg attttgtacg cgacggaccc    | 300 |
| 10 | gccatgtaat cgtcgttcgt gacgatgcaa gggttcacgc acatctacac acactcactc    | 360 |
|    | acacaactag tgtaactcgt atagaattcg gtgtcgacct ggaccttggt tgactgggtg    | 420 |
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|    | 1 5 10 15  |     |
|    | Ala Glu Xaa Gly Phe Pro Pro Lys Pro Phe Val Asp Trp Phe Cys          |     |
|    | 20 25 30   |     |
| 30 | Gly Gly Phe Gln Tyr Gln Val Asp His His Leu Phe Pro Ser Leu          |     |
|    | 35 40 45   |     |
|    | Pro Arg His Asn Leu Ala Lys Thr His Ala Leu Val Glu Ser Phe          |     |
|    | 50 55 60   |     |
| 35 | Cys Lys Glu Trp Gly Val Gln Tyr His Glu Ala Asp Leu Val Asp          |     |
|    | 65 70 75   |     |
|    | Gly Thr Met Glu Val Leu His His Leu Gly Ser Val Ala Gly Glu          |     |
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|    | Phe Val Val Asp Phe Val Arg Asp Gly Pro Ala Met                      |     |
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|    | cttgggtaca cgccggggca gtcgttgggc atgtacttgt gcgcctttgg tctcggtgc     | 120 |
|    | atttacattt ttctgcagtt cgccgtaagt cacaccatt tgcccgtag caaccggag       | 180 |
| 55 | gatcagctgc attggctcga gtacgcgcgg accacactgt gaacatcagc accaagtcgt    | 240 |
|    | ggtttgtcac atggtggatg tcgaacctca actttcagat cgagcaccac cttttcccca    | 300 |
|    | cggcgcccc gttccgtttc aaggagatca gccgcgcgt cgaggccctc ttcaagcgcc      | 360 |
| 60 | acggtctccc ttactacgac atgccctaca cgagcgccgt ctccaccacc tttgccaacc    | 420 |
|    | tctactcgt cggccattcc gtcggcgacg ccaagcgga ctagcctctt ttctagacc       | 480 |
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 35 40 45  
 15 Val Arg Arg Lys Ser His Pro Phe Ala Arg Glu Gln Pro Gly Gly  
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 Ser Thr Lys Ser Trp Phe Val Thr Trp Trp Met Ser Asn Leu Asn  
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 Gln Gly Arg Cys Gly Trp Val Met His Glu Met Gly His Gly Ser  
 65 70 75  
 70 Phe Thr Gly Val Ile Trp Leu Asp Asp Arg Leu Cys Glu Phe Phe

|    |             |            |            |            |              |            |     |     |     |     |     |     |     |     |      |
|----|-------------|------------|------------|------------|--------------|------------|-----|-----|-----|-----|-----|-----|-----|-----|------|
|    |             |            |            |            | 65           |            |     |     |     | 70  |     |     |     |     | 75   |
|    | Tyr         | Gly        | Val        | Gly        | Cys          | Gly        | Met | Ser | Gly | His | Tyr | Trp | Lys | Asn | Gln  |
|    |             |            |            |            | 80           |            |     |     |     | 85  |     |     |     |     | 90   |
| 5  | His         | Ser        | Lys        | His        | His          | Ala        | Ala | Pro | Asn | Arg | Leu | Glu | His | Asp | Val  |
|    |             |            |            |            | 95           |            |     |     |     | 100 |     |     |     |     | 105  |
|    | Asp         | Leu        | Asn        | Thr        | Leu          | Pro        | Leu | Val | Ala | Phe | Asn | Glu | Arg | Val | Val  |
|    |             |            |            |            | 110          |            |     |     |     | 115 |     |     |     |     | 120  |
|    | Arg         | Lys        | Val        | Arg        | Pro          |            |     |     |     |     |     |     |     |     |      |
|    |             |            |            |            | 125          |            |     |     |     |     |     |     |     |     |      |
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| 20 | acctgatccc  | aatttgatat | ggattataat | tatgatgggt | ctcaccagc    | tgggtgcatt |     |     |     |     |     |     |     |     | 120  |
|    | ttacatagta  | aaagacttgg | actggaaatg | ggatcatatt | ggggcctatg   | cgtttggcag |     |     |     |     |     |     |     |     | 180  |
| 25 | ttgcattaac  | cactcaatga | ctctggctat | tcatgagatt | gcccacaatg   | ctgcctttgg |     |     |     |     |     |     |     |     | 240  |
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Met Met Val Leu Thr Gln Leu Gly Ala Phe Tyr Ile Val Lys Asp
     35     40     45
Leu Asp Trp Lys Trp Val Ile Phe Gly Ala Tyr Ala Phe Gly Ser
     50     55     60
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Asn Ala Ala Phe Gly Asn Cys Lys Ala Met Trp Asn Arg Trp Phe
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Gly Met Phe Ala Asn Leu Pro Ile Gly Ile Pro Tyr Ser Ile Ser
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Phe Lys Arg Tyr His Met Asp His His Arg Tyr Leu Gly Ala Asp
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Gly Val Asp Val Asp Ile Pro Thr Asp Phe Glu Gly Trp Phe Phe
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Phe Tyr Ala Phe Arg Pro Leu Phe Ile Asn Pro Lys Pro Ile Thr
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Tyr Leu Glu Val Ile Asn Thr Val Ala Gln Val Thr Phe Asp Ile
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Leu Ile Tyr Tyr Phe Leu Gly Ile Lys Ser Leu Val Tyr Met Leu
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Ala Ala Ser Leu Leu Gly Leu Gly Leu His Pro Ile Ser Gly His
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Phe Ile Ala Glu His Tyr Met Phe Leu Lys Gly His Glu Thr Tyr
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Ser Tyr Tyr Gly Pro Leu Asn Leu Leu Thr Phe Asn Val Gly Tyr
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|----|-------|-----|-----|-----|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | His   | Asn | Glu | His | His          | Asp | Phe | Pro | Asn | Ile | Pro | Gly | Lys | Ser | Leu |     |
|    |       |     |     |     | 245          |     |     |     |     | 250 |     |     |     |     |     | 255 |
|    | Pro   | Leu | Val | Arg | Lys          | Ile | Ala | Ala | Glu | Tyr | Tyr | Asp | Asn | Leu | Pro |     |
|    |       |     |     |     | 260          |     |     |     |     | 265 |     |     |     |     |     | 270 |
| 5  | His   | Tyr | Asn | Ser | Trp          | Ile | Lys | Val | Leu | Tyr | Asp | Phe | Val | Met | Asp |     |
|    |       |     |     |     | 275          |     |     |     |     | 280 |     |     |     |     |     | 285 |
|    | Asp   | Thr | Ile | Ser | Pro          | Tyr | Ser | Arg | Met | Lys | Arg | His | Gln | Lys | Gly |     |
|    |       |     |     |     | 290          |     |     |     |     | 295 |     |     |     |     |     | 300 |
| 10 | Glu   | Met | Val | Leu | Glu          | Xaa | Ile | Ser | Leu | Val | Pro | Lys | Gly | Phe | Phe |     |
|    |       |     |     |     | 305          |     |     |     |     | 310 |     |     |     |     |     | 315 |
|    | Ser   | Lys | Thr | Leu | Asp          | Asp | Lys | Met | Glu | Phe | Leu | His | Tyr | Xaa | Thr |     |
|    |       |     |     |     | 320          |     |     |     |     | 325 |     |     |     |     |     | 330 |
|    | Xaa   | Asp | Gln | Xaa | Cys          | Ser | Glu | Ala | Pro | Leu | Ala | Gln | Phe | Gln | Ser |     |
|    |       |     |     |     | 335          |     |     |     |     | 340 |     |     |     |     |     | 345 |
| 15 | Lys   | Ser | Ser | Val | Ile          | Pro | Arg | Ser | Glu | Ser | Gly | Phe | Xaa | Thr | Val |     |
|    |       |     |     |     | 350          |     |     |     |     | 355 |     |     |     |     |     | 360 |
|    | Ser   | Leu | Thr | Leu | Tyr          | Cys | Ser | Val | Ser | Leu | Thr | Gly | Asn | Leu | Xaa |     |
|    |       |     |     |     | 365          |     |     |     |     | 370 |     |     |     |     |     | 375 |
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|    | Phe   | Ile | Ser | Ile | Ser          | Phe | Lys | Lys | Leu | Leu | Lys | Ser | Tyr | Phe | Ala |     |
|    |       |     |     |     | 400          |     |     |     |     | 405 |     |     |     |     |     | 410 |
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|    | Tyr   | Leu | Leu | Arg | Pro          | Leu | Leu | Pro | His | Leu | Cys | Ala | Thr | Ile | Gly |     |
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|    |       |     |     |     | 20           |     |     |     |     | 25  |     |     |     |     | 30  |     |
|    | Ser   | Asn | Trp | Phe | Val          | Trp | Val | Thr | Gln | Met | Asn | His | Ile | Pro | Met |     |
|    |       |     |     |     | 35           |     |     |     |     | 40  |     |     |     |     | 45  |     |
| 40 | His   | Ile | Asp | His | Asp          | Arg | Asn | Met | Asp | Trp | Val | Ser | Thr | Gln | Leu |     |
|    |       |     |     |     | 50           |     |     |     |     | 55  |     |     |     |     | 60  |     |
|    | Gln   | Ala | Thr | Cys | Asn          | Val | His | Lys | Ser | Ala | Phe | Asn | Asp | Trp | Phe |     |
|    |       |     |     |     | 65           |     |     |     |     | 70  |     |     |     |     | 75  |     |
|    | Ser   | Gly | His | Leu | Asn          | Phe | Gln | Ile | Glu | His | His | Leu | Phe | Pro | Thr |     |
|    |       |     |     |     | 80           |     |     |     |     | 85  |     |     |     |     | 90  |     |
| 45 | Met   | Pro | Arg | His | Asn          | Tyr | His | Lys | Val | Ala | Pro | Leu | Val | Gln | Ser |     |
|    |       |     |     |     | 95           |     |     |     |     | 100 |     |     |     |     | 105 |     |
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|    |       |     |     |     | 110          |     |     |     |     | 115 |     |     |     |     | 120 |     |
| 50 | Ser   | Ala | Phe | Ala | Asp          | Ile | Ile | His | Ser | Leu | Lys | Glu | Ser | Gly | Gln |     |
|    |       |     |     |     | 125          |     |     |     |     | 130 |     |     |     |     | 135 |     |
|    | Leu   | Trp | Leu | Asp | Ala          | Tyr | Leu | His | Gln | Xaa | Gln | Gln | Pro | Pro | Cys |     |
|    |       |     |     |     | 140          |     |     |     |     | 145 |     |     |     |     | 150 |     |
|    | Pro   | Val | Trp | Lys | Lys          | Arg | Arg | Lys | Thr | Leu | Glu | Pro | Arg | Gln | Arg |     |
|    |       |     |     |     | 155          |     |     |     |     | 160 |     |     |     |     | 165 |     |
| 55 | Gly   | Ala | Xaa | Gly | Thr          | Met | Pro | Leu | Xaa | Phe | Asn | Thr | Gln | Arg | Gly |     |
|    |       |     |     |     | 170          |     |     |     |     | 175 |     |     |     |     | 180 |     |
|    | Leu   | Gly | Leu | Gly | Thr          | Xaa | Ser | Leu | Xaa | Leu | Lys | Leu | Leu | Pro | Phe |     |
|    |       |     |     |     | 185          |     |     |     |     | 190 |     |     |     |     | 195 |     |
| 60 | Ile   | Phe | Xaa | Pro | Gln          | Phe | Xaa | Asp | Pro | Lys | Trp | Gly | Val | Asp | Thr |     |
|    |       |     |     |     | 200          |     |     |     |     | 205 |     |     |     |     | 210 |     |
|    | Glu   | Val | Pro | Arg | Arg          | Glu | Gly | Ala |     |     |     |     |     |     |     |     |
|    |       |     |     |     | 215          |     |     |     |     |     |     |     |     |     |     |     |
| 65 |       |     |     |     |              |     |     |     |     |     |     |     |     |     |     |     |
|    | <210> |     |     |     | 30           |     |     |     |     |     |     |     |     |     |     |     |
|    | <211> |     |     |     | 71           |     |     |     |     |     |     |     |     |     |     |     |
|    | <212> |     |     |     | PRT          |     |     |     |     |     |     |     |     |     |     |     |
|    | <213> |     |     |     | Homo sapiens |     |     |     |     |     |     |     |     |     |     |     |
| 70 |       |     |     |     |              |     |     |     |     |     |     |     |     |     |     |     |
|    | <400> |     |     |     | 30           |     |     |     |     |     |     |     |     |     |     |     |

5 Val Phe Tyr Phe Gly Asn Gly Trp Ile Pro Thr Leu Ile Thr Ala  
 1 5 10 15  
 Phe Val Leu Ala Thr Ser Gln Ala Gln Ala Gly Trp Leu Gln His  
 20 25 30  
 Asp Tyr Gly His Leu Ser Val Tyr Arg Lys Pro Lys Trp Asn His  
 35 40 45  
 Leu Val His Lys Phe Val Ile Gly His Leu Lys Gly Ala Ser Ala  
 50 55 60  
 10 Asn Trp Trp Asn His Arg His Phe Gln His His Ala Lys Pro Asn  
 65 70 75  
 Leu Gly Glu Trp Gln Pro Ile Glu Tyr Gly Lys Xaa  
 80 85  
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 Arg Ser Gly Cys Glu Glu Arg Trp Leu Val Ile Asp Arg Lys Val  
 20 25 30  
 Tyr Asn Ile Ser Glu Phe Thr Arg Arg His Pro Gly Gly Ser Arg  
 35 40 45  
 Val Ile Ser His Tyr Ala Gly Gln Asp Ala Thr Asp Pro Phe Val  
 50 55 60  
 Ala Phe His Ile Asn Lys Gly Leu Val Lys Lys Tyr Met Asn Ser  
 65 70 75  
 Leu Leu Ile Gly Glu Leu Ser Pro Glu Gln Pro Ser Phe Glu Pro  
 80 85 90  
 35 Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe Arg Glu Leu Arg Ala  
 95 100 105  
 Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn His Val Phe Phe  
 110 115 120  
 40 Leu Leu Tyr Leu Leu His Ile Leu Leu Leu Asp Gly Ala Ala Trp  
 125 130 135  
 Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe Leu Leu  
 140 145 150  
 Cys Ala Val Leu Leu Ser Ala Val Gln Ala Gln Ala Gly Trp Leu  
 155 160 165  
 45 Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr Ser Lys Trp  
 170 175 180  
 Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys Gly Ala  
 185 190 195  
 50 Pro Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala Lys  
 200 205 210  
 Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe  
 215 220 225  
 Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln  
 230 235 240  
 55 Lys Lys Lys Tyr Met Pro Tyr Asn His Gln His Xaa Tyr Phe Phe  
 245 250 255  
 Leu Ile Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr  
 260 265 270  
 60 Ile Phe Tyr Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala  
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 Trp Ile Ser Lys Gln Glu Tyr Asp Glu Ala Gly Leu Pro Leu Ser  
 290 295 300  
 Thr Ala Asn Ala Ser Lys  
 305  
 65  
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 70

&lt;400&gt; 32

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|    | His | Leu | Lys | Gly | Ala | Ser | Ala | Asn | Trp | Trp | Asn | His | Arg | His | Phe |  |
| 5  | 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |  |
|    | Gln | His | His | Ala | Lys | Pro | Asn | Ile | Phe | His | Lys | Asp | Pro | Asp | Val |  |
|    |     |     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |  |
|    | Asn | Met | Leu | His | Val | Phe | Val | Leu | Gly | Glu | Trp | Gln | Pro | Ile | Glu |  |
|    |     |     |     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |  |
| 10 | Tyr | Gly | Lys | Lys | Lys | Leu | Lys | Tyr | Leu | Pro | Tyr | Asn | His | Gln | His |  |
|    |     |     |     |     | 50  |     |     |     |     | 55  |     |     |     |     | 60  |  |
|    | Glu | Tyr | Phe | Phe | Leu | Ile | Gly | Pro | Pro | Leu | Leu | Ile | Pro | Met | Tyr |  |
|    |     |     |     |     | 65  |     |     |     |     | 70  |     |     |     |     | 75  |  |
|    | Phe | Gln | Tyr | Gln | Ile | Ile | Met | Thr | Met | Ile | Val | His | Lys | Asn | Trp |  |
| 15 |     |     |     |     | 80  |     |     |     |     | 85  |     |     |     |     | 90  |  |
|    | Val | Asp | Leu | Ala | Trp | Ala | Val | Ser | Tyr | Tyr | Ile | Arg | Phe | Phe | Ile |  |
|    |     |     |     |     | 95  |     |     |     |     | 100 |     |     |     |     | 105 |  |
|    | Thr | Tyr | Ile | Pro | Phe | Tyr | Gly | Ile | Leu | Gly | Ala | Leu | Leu | Phe | Leu |  |
|    |     |     |     |     | 110 |     |     |     |     | 115 |     |     |     |     | 120 |  |
| 20 | Asn | Phe | Ile | Arg | Phe | Leu | Glu | Ser | His | Trp | Phe | Val | Trp | Val | Thr |  |
|    |     |     |     |     | 125 |     |     |     |     | 130 |     |     |     |     | 135 |  |
|    | Gln | Met | Asn | His | Ile | Val | Met | Glu | Ile | Asp | Gln | Glu | Ala | Tyr | Arg |  |
|    |     |     |     |     | 140 |     |     |     |     | 145 |     |     |     |     | 150 |  |
|    | Asp | Trp | Phe | Ser | Ser | Gln | Leu | Thr | Ala | Thr | Cys | Asn | Val | Glu | Gln |  |
| 25 |     |     |     |     | 155 |     |     |     |     | 160 |     |     |     |     | 165 |  |
|    | Ser | Phe | Phe | Asn | Asp | Trp | Phe | Ser | Gly | His | Leu | Asn | Phe | Gln | Ile |  |
|    |     |     |     |     | 170 |     |     |     |     | 175 |     |     |     |     | 180 |  |
|    | Glu | His | His | Leu | Phe | Pro | Thr | Met | Pro | Arg | His | Asn | Leu | His | Lys |  |
|    |     |     |     |     | 185 |     |     |     |     | 190 |     |     |     |     | 195 |  |
| 30 | Ile | Ala | Pro | Leu | Val | Lys | Ser | Leu | Cys | Ala | Lys | His | Gly | Ile | Glu |  |
|    |     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |     | 210 |  |
|    | Tyr | Gln | Glu | Lys | Pro | Leu | Leu | Arg | Ala | Leu | Leu | Asp | Ile | Ile | Arg |  |
|    |     |     |     |     | 215 |     |     |     |     | 220 |     |     |     |     | 225 |  |
|    | Ser | Leu | Lys | Lys | Ser | Gly | Lys | Leu | Trp | Leu | Asp | Ala | Tyr | Leu | His |  |
| 35 |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     |     | 240 |  |
|    | Lys | Xaa | Ser | His | Ser | Pro | Arg | Asp | Thr | Val | Gly | Lys | Gly | Cys | Arg |  |
|    |     |     |     |     | 245 |     |     |     |     | 250 |     |     |     |     | 255 |  |
|    | Trp | Gly | Asp | Gly | Gln | Arg | Asn | Asp | Gly | Leu | Leu | Phe | Xaa | Gly | Val |  |
|    |     |     |     |     | 260 |     |     |     |     | 265 |     |     |     |     | 270 |  |
| 40 | Ser | Glu | Arg | Leu | Val | Tyr | Ala | Leu | Leu | Thr | Asp | Pro | Met | Leu | Asp |  |
|    |     |     |     |     | 275 |     |     |     |     | 280 |     |     |     |     | 285 |  |
|    | Leu | Ser | Pro | Phe | Leu | Leu | Ser | Phe | Phe | Ser | Ser | His | Leu | Pro | His |  |
|    |     |     |     |     | 290 |     |     |     |     | 295 |     |     |     |     | 300 |  |
|    | Ser | Thr | Leu | Pro | Ser | Trp | Asp | Leu | Pro | Ser | Leu | Ser | Arg | Gln | Pro |  |
| 45 |     |     |     |     | 305 |     |     |     |     | 310 |     |     |     |     | 315 |  |
|    | Ser | Ala | Met | Ala | Leu | Pro | Val | Pro | Pro | Ser | Pro | Phe | Phe | Gln | Gly |  |
|    |     |     |     |     | 320 |     |     |     |     | 325 |     |     |     |     | 330 |  |
|    | Ala | Glu | Arg | Trp | Pro | Pro | Gly | Val | Ala | Leu | Ser | Tyr | Leu | His | Ser |  |
|    |     |     |     |     | 335 |     |     |     |     | 340 |     |     |     |     | 345 |  |
| 50 | Leu | Pro | Leu | Lys | Met | Gly | Gly | Asp | Gln | Arg | Ser | Met | Gly | Leu | Ala |  |
|    |     |     |     |     | 350 |     |     |     |     | 355 |     |     |     |     | 360 |  |
|    | Cys | Glu | Ser | Pro | Leu | Ala | Ala | Trp | Ser | Leu | Gly | Ile | Thr | Pro | Ala |  |
|    |     |     |     |     | 365 |     |     |     |     | 370 |     |     |     |     | 375 |  |
|    | Leu | Val | Leu | Gln | Met | Leu | Leu | Gly | Phe | Ile | Gly | Ala | Gly | Pro | Ser |  |
| 55 |     |     |     |     | 380 |     |     |     |     | 385 |     |     |     |     | 390 |  |
|    | Arg | Ala | Gly | Pro | Leu | Thr | Leu | Pro | Ala | Trp | Leu | His | Ser | Pro | Xaa |  |
|    |     |     |     |     | 400 |     |     |     |     | 405 |     |     |     |     | 410 |  |
|    | Arg | Leu | Pro | Leu | Val | His | Pro | Phe | Ile | Glu | Arg | Pro | Ala | Leu | Leu |  |
|    |     |     |     |     | 415 |     |     |     |     | 420 |     |     |     |     | 425 |  |
| 60 | Gln | Ser | Ser | Gly | Leu | Pro | Pro | Ala | Ala | Arg | Leu | Ser | Thr | Arg | Gly |  |
|    |     |     |     |     | 430 |     |     |     |     | 435 |     |     |     |     | 440 |  |
|    | Leu | Ser | Xaa | Asp | Val | Gln | Gly | Pro | Arg | Pro | Ala | Gly | Thr | Ala | Ser |  |
|    |     |     |     |     | 445 |     |     |     |     | 450 |     |     |     |     | 455 |  |
|    | Pro | Asn | Leu | Gly | Pro | Trp | Lys | Ser | Pro | Pro | Pro | His | His | Xaa | Ser |  |
| 65 |     |     |     |     | 460 |     |     |     |     | 465 |     |     |     |     | 470 |  |
|    | Ala | Leu | Thr | Leu | Gly | Phe | His | Gly | Pro | His | Ser | Thr | Ala | Ser | Pro |  |
|    |     |     |     |     | 475 |     |     |     |     | 480 |     |     |     |     | 485 |  |
|    | Thr | Xaa | Ala | Cys | Asp | Leu | Gly | Thr | Lys | Gly | Gly | Val | Pro | Arg | Leu |  |
|    |     |     |     |     | 490 |     |     |     |     | 495 |     |     |     |     | 500 |  |
| 70 | Leu | Xaa | Leu | Ser | Arg | Gly | Ser | Gly | His | Val | Gln | Gly | Gly | Ala | Gly |  |

|    |       |     |     |              |     |     |     |     |     |     |     |     |     |     |     |
|----|-------|-----|-----|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    |       |     |     | 505          |     |     |     |     | 510 |     |     |     | 515 |     |     |
|    | Trp   | Pro | Gly | Gly          | Ser | Ala | His | Pro | Pro | Ala | Phe | Pro | Gln | Gly | Val |
|    |       |     |     | 520          |     |     |     |     |     | 525 |     |     |     |     | 530 |
| 5  | Leu   | Arg | Ser | Lys          | Ile | Leu | Glu | Gln | Ser | Asp | Pro | Ser | Pro | Lys | Ala |
|    |       |     |     | 535          |     |     |     |     |     | 540 |     |     |     |     | 545 |
|    | Leu   | Leu | Ser | Ala          | Gly | Gln | Cys | Gln | Pro | Ile | Pro | Gly | His | Leu | Ala |
|    |       |     |     | 550          |     |     |     |     |     | 555 |     |     |     |     | 560 |
|    | Pro   | Gly | Asp | Val          | Gly | Pro | Xaa |     |     |     |     |     |     |     |     |
|    |       |     |     | 565          |     |     |     |     |     |     |     |     |     |     |     |
| 10 |       |     |     |              |     |     |     |     |     |     |     |     |     |     |     |
|    | <210> |     |     | 33           |     |     |     |     |     |     |     |     |     |     |     |
|    | <211> |     |     | 619          |     |     |     |     |     |     |     |     |     |     |     |
|    | <212> |     |     | PRT          |     |     |     |     |     |     |     |     |     |     |     |
| 15 | <213> |     |     | Homo sapiens |     |     |     |     |     |     |     |     |     |     |     |
|    | <400> |     |     | 33           |     |     |     |     |     |     |     |     |     |     |     |
| 20 | Val   | Phe | Tyr | Phe          | Gly | Asn | Gly | Trp | Ile | Pro | Thr | Leu | Ile | Thr | Ala |
|    | 1     |     |     |              | 5   |     |     |     |     | 10  |     |     |     |     | 15  |
|    | Phe   | Val | Leu | Ala          | Thr | Ser | Gln | Ala | Gln | Ala | Gly | Trp | Leu | Gln | His |
|    |       |     |     |              | 20  |     |     |     |     | 25  |     |     |     |     | 30  |
|    | Asp   | Tyr | Gly | His          | Leu | Ser | Val | Tyr | Arg | Lys | Pro | Lys | Trp | Asn | His |
|    |       |     |     |              | 35  |     |     |     |     | 40  |     |     |     |     | 45  |
| 25 | Leu   | Val | His | Lys          | Phe | Val | Ile | Gly | His | Leu | Lys | Gly | Ala | Ser | Ala |
|    |       |     |     |              | 50  |     |     |     |     | 55  |     |     |     |     | 60  |
|    | Asn   | Trp | Trp | Asn          | His | Arg | His | Phe | Gln | His | His | Ala | Lys | Pro | Asn |
|    |       |     |     |              | 65  |     |     |     |     | 70  |     |     |     |     | 75  |
|    | Ile   | Phe | His | Lys          | Asp | Pro | Asp | Val | Asn | Met | Leu | His | Val | Phe | Val |
| 30 |       |     |     |              | 80  |     |     |     |     | 85  |     |     |     |     | 90  |
|    | Leu   | Gly | Glu | Trp          | Gln | Pro | Ile | Glu | Tyr | Gly | Lys | Lys | Lys | Leu | Lys |
|    |       |     |     |              | 95  |     |     |     |     | 100 |     |     |     |     | 105 |
|    | Tyr   | Leu | Pro | Tyr          | Asn | His | Gln | His | Glu | Tyr | Phe | Phe | Leu | Ile | Gly |
|    |       |     |     |              | 110 |     |     |     |     | 115 |     |     |     |     | 120 |
| 35 | Pro   | Pro | Leu | Leu          | Ile | Pro | Met | Tyr | Phe | Gln | Tyr | Gln | Ile | Ile | Met |
|    |       |     |     |              | 125 |     |     |     |     | 130 |     |     |     |     | 135 |
|    | Thr   | Met | Ile | Val          | His | Lys | Asn | Trp | Val | Asp | Leu | Ala | Trp | Ala | Val |
|    |       |     |     |              | 140 |     |     |     |     | 145 |     |     |     |     | 150 |
| 40 | Ser   | Tyr | Tyr | Ile          | Arg | Phe | Phe | Ile | Thr | Tyr | Ile | Pro | Phe | Tyr | Gly |
|    |       |     |     |              | 155 |     |     |     |     | 160 |     |     |     |     | 165 |
|    | Ile   | Leu | Gly | Ala          | Leu | Leu | Phe | Leu | Asn | Phe | Ile | Arg | Phe | Leu | Glu |
|    |       |     |     |              | 170 |     |     |     |     | 175 |     |     |     |     | 180 |
|    | Ser   | His | Trp | Phe          | Val | Trp | Val | Thr | Gln | Met | Asn | His | Ile | Val | Met |
|    |       |     |     |              | 185 |     |     |     |     | 190 |     |     |     |     | 195 |
| 45 | Glu   | Ile | Asp | Gln          | Glu | Ala | Tyr | Arg | Asp | Trp | Phe | Ser | Ser | Gln | Leu |
|    |       |     |     |              | 200 |     |     |     |     | 205 |     |     |     |     | 210 |
|    | Thr   | Ala | Thr | Cys          | Asn | Val | Glu | Gln | Ser | Phe | Phe | Asn | Asp | Trp | Phe |
|    |       |     |     |              | 215 |     |     |     |     | 220 |     |     |     |     | 225 |
|    | Ser   | Gly | His | Leu          | Asn | Phe | Gln | Ile | Glu | His | His | Leu | Phe | Pro | Thr |
| 50 |       |     |     |              | 230 |     |     |     |     | 235 |     |     |     |     | 240 |
|    | Met   | Pro | Arg | His          | Asn | Leu | His | Lys | Ile | Ala | Pro | Leu | Val | Lys | Ser |
|    |       |     |     |              | 245 |     |     |     |     | 250 |     |     |     |     | 255 |
|    | Leu   | Cys | Ala | Lys          | His | Gly | Ile | Glu | Tyr | Gln | Glu | Lys | Pro | Leu | Leu |
|    |       |     |     |              | 260 |     |     |     |     | 265 |     |     |     |     | 270 |
| 55 | Arg   | Ala | Leu | Leu          | Asp | Ile | Ile | Arg | Ser | Leu | Lys | Lys | Ser | Gly | Lys |
|    |       |     |     |              | 275 |     |     |     |     | 280 |     |     |     |     | 285 |
|    | Leu   | Trp | Leu | Asp          | Ala | Tyr | Leu | His | Lys | Xaa | Ser | His | Ser | Pro | Arg |
|    |       |     |     |              | 290 |     |     |     |     | 295 |     |     |     |     | 300 |
|    | Asp   | Thr | Val | Gly          | Lys | Gly | Cys | Arg | Trp | Gly | Asp | Gly | Gln | Arg | Asn |
| 60 |       |     |     |              | 305 |     |     |     |     | 310 |     |     |     |     | 315 |
|    | Asp   | Gly | Leu | Leu          | Phe | Xaa | Gly | Val | Ser | Glu | Arg | Leu | Val | Tyr | Ala |
|    |       |     |     |              | 320 |     |     |     |     | 325 |     |     |     |     | 330 |
|    | Leu   | Leu | Thr | Asp          | Pro | Met | Leu | Asp | Leu | Ser | Pro | Phe | Leu | Leu | Ser |
|    |       |     |     |              | 335 |     |     |     |     | 340 |     |     |     |     | 345 |
| 65 | Phe   | Phe | Ser | Ser          | His | Leu | Pro | His | Ser | Thr | Leu | Pro | Ser | Trp | Asp |
|    |       |     |     |              | 350 |     |     |     |     | 355 |     |     |     |     | 360 |
|    | Leu   | Pro | Ser | Leu          | Ser | Arg | Gln | Pro | Ser | Ala | Met | Ala | Leu | Pro | Val |
|    |       |     |     |              | 365 |     |     |     |     | 370 |     |     |     |     | 375 |
| 70 | Pro   | Pro | Ser | Pro          | Phe | Phe | Gln | Gly | Ala | Glu | Arg | Trp | Pro | Pro | Gly |
|    |       |     |     |              | 380 |     |     |     |     | 385 |     |     |     |     | 390 |

|    |                    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|--------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | Val                | Ala | Leu | Ser | Tyr | Leu | His | Ser | Leu | Pro | Leu | Lys | Met | Gly | Gly |
|    |                    |     |     |     | 400 |     |     |     |     | 405 |     |     |     |     | 410 |
|    | Asp                | Gln | Arg | Ser | Met | Gly | Leu | Ala | Cys | Glu | Ser | Pro | Leu | Ala | Ala |
|    |                    |     |     |     | 415 |     |     |     |     | 420 |     |     |     |     | 425 |
| 5  | Trp                | Ser | Leu | Gly | Ile | Thr | Pro | Ala | Leu | Val | Leu | Gln | Met | Leu | Leu |
|    |                    |     |     |     | 430 |     |     |     |     | 435 |     |     |     |     | 440 |
|    | Gly                | Phe | Ile | Gly | Ala | Gly | Pro | Ser | Arg | Ala | Gly | Pro | Leu | Thr | Leu |
|    |                    |     |     |     | 445 |     |     |     |     | 450 |     |     |     |     | 455 |
|    | Pro                | Ala | Trp | Leu | His | Ser | Pro | Xaa | Arg | Leu | Pro | Leu | Val | His | Pro |
| 10 |                    |     |     |     | 460 |     |     |     |     | 465 |     |     |     |     | 470 |
|    | Phe                | Ile | Glu | Arg | Pro | Ala | Leu | Leu | Gln | Ser | Ser | Gly | Leu | Pro | Pro |
|    |                    |     |     |     | 475 |     |     |     |     | 480 |     |     |     |     | 485 |
|    | Ala                | Ala | Arg | Leu | Ser | Thr | Arg | Gly | Leu | Ser | Xaa | Asp | Val | Gln | Gly |
|    |                    |     |     |     | 490 |     |     |     |     | 495 |     |     |     |     | 500 |
| 15 | Pro                | Arg | Pro | Ala | Gly | Thr | Ala | Ser | Pro | Asn | Leu | Gly | Pro | Trp | Lys |
|    |                    |     |     |     | 505 |     |     |     |     | 510 |     |     |     |     | 515 |
|    | Ser                | Pro | Pro | Pro | His | His | Xaa | Ser | Ala | Leu | Thr | Leu | Gly | Phe | His |
|    |                    |     |     |     | 520 |     |     |     |     | 525 |     |     |     |     | 530 |
|    | Gly                | Pro | His | Ser | Thr | Ala | Ser | Pro | Thr | Xaa | Ala | Cys | Asp | Leu | Gly |
| 20 |                    |     |     |     | 535 |     |     |     |     | 540 |     |     |     |     | 545 |
|    | Thr                | Lys | Gly | Gly | Val | Pro | Arg | Leu | Leu | Xaa | Leu | Ser | Arg | Gly | Ser |
|    |                    |     |     |     | 550 |     |     |     |     | 555 |     |     |     |     | 560 |
|    | Gly                | His | Val | Gln | Gly | Gly | Ala | Gly | Trp | Pro | Gly | Gly | Ser | Ala | His |
|    |                    |     |     |     | 565 |     |     |     |     | 570 |     |     |     |     | 575 |
| 25 | Pro                | Pro | Ala | Phe | Pro | Gln | Gly | Val | Leu | Arg | Ser | Lys | Ile | Leu | Glu |
|    |                    |     |     |     | 580 |     |     |     |     | 585 |     |     |     |     | 590 |
|    | Gln                | Ser | Asp | Pro | Ser | Pro | Lys | Ala | Leu | Leu | Ser | Ala | Gly | Gln | Cys |
|    |                    |     |     |     | 595 |     |     |     |     | 600 |     |     |     |     | 605 |
| 30 | Gln                | Pro | Ile | Pro | Gly | His | Leu | Ala | Pro | Gly | Asp | Val | Gly | Pro | Xaa |
|    |                    |     |     |     | 610 |     |     |     |     | 615 |     |     |     |     | 620 |
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| 35 | <212> PRT          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|    | <213> Homo sapiens |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|    | <400> 34           |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 40 | Gln                | Gly | Pro | Thr | Pro | Arg | Tyr | Phe | Thr | Trp | Asp | Glu | Val | Ala | Gln |
|    | 1                  |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |
|    | Arg                | Ser | Gly | Cys | Glu | Glu | Arg | Trp | Leu | Val | Ile | Asp | Arg | Lys | Val |
|    |                    |     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |
| 45 | Tyr                | Asn | Ile | Ser | Glu | Phe | Thr | Arg | Arg | His | Pro | Gly | Gly | Ser | Arg |
|    |                    |     |     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |
|    | Val                | Ile | Ser | His | Tyr | Ala | Gly | Gln | Asp | Ala | Thr | Asp | Pro | Phe | Val |
|    |                    |     |     |     | 50  |     |     |     |     | 55  |     |     |     |     | 60  |
|    | Ala                | Phe | His | Ile | Asn | Lys | Gly | Leu | Val | Lys | Lys | Tyr | Met | Asn | Ser |
|    |                    |     |     |     | 65  |     |     |     |     | 70  |     |     |     |     | 75  |
| 50 | Leu                | Leu | Ile | Gly | Glu | Leu | Ser | Pro | Glu | Gln | Pro | Ser | Phe | Glu | Pro |
|    |                    |     |     |     | 80  |     |     |     |     | 85  |     |     |     |     | 90  |
|    | Thr                | Lys | Asn | Lys | Glu | Leu | Thr | Asp | Glu | Phe | Arg | Glu | Leu | Arg | Ala |
|    |                    |     |     |     | 95  |     |     |     |     | 100 |     |     |     |     | 105 |
| 55 | Thr                | Val | Glu | Arg | Met | Gly | Leu | Met | Lys | Ala | Asn | His | Val | Phe | Phe |
|    |                    |     |     |     | 110 |     |     |     |     | 115 |     |     |     |     | 120 |
|    | Leu                | Leu | Tyr | Leu | Leu | His | Ile | Leu | Leu | Leu | Asp | Gly | Ala | Ala | Trp |
|    |                    |     |     |     | 125 |     |     |     |     | 130 |     |     |     |     | 135 |
|    | Leu                | Thr | Leu | Trp | Val | Phe | Gly | Thr | Ser | Phe | Leu | Pro | Phe | Leu | Leu |
|    |                    |     |     |     | 140 |     |     |     |     | 145 |     |     |     |     | 150 |
| 60 | Cys                | Ala | Val | Leu | Leu | Ser | Ala | Val | Gln | Gln | Ala | Gln | Ala | Gly | Trp |
|    |                    |     |     |     | 155 |     |     |     |     | 160 |     |     |     |     | 165 |
|    | Leu                | Gln | His | Asp | Tyr | Gly | His | Leu | Ser | Val | Tyr | Arg | Lys | Pro | Lys |
|    |                    |     |     |     | 170 |     |     |     |     | 175 |     |     |     |     | 180 |
| 65 | Trp                | Asn | His | Leu | Val | His | Lys | Phe | Val | Ile | Gly | His | Leu | Lys | Gly |
|    |                    |     |     |     | 185 |     |     |     |     | 190 |     |     |     |     | 195 |
|    | Ala                | Ser | Ala | Asn | Trp | Trp | Asn | His | Arg | His | Phe | Gln | His | His | Ala |
|    |                    |     |     |     | 200 |     |     |     |     | 205 |     |     |     |     | 210 |
|    | Lys                | Pro | Asn | Ile | Phe | His | Lys | Asp | Pro | Asp | Val | Asn | Met | Leu | His |
|    |                    |     |     |     | 215 |     |     |     |     | 220 |     |     |     |     | 225 |
| 70 | Val                | Phe | Val | Leu | Gly | Glu | Trp | Gln | Pro | Ile | Glu | Tyr | Gly | Lys | Lys |

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    |     |     |     | 230 |     |     |     |     | 235 |     |     |     |     | 240 |
|    | Lys | Leu | Lys | Tyr | Leu | Pro | Tyr | Asn | His | Gln | His | Glu | Tyr | Phe |
|    |     |     |     | 245 |     |     |     |     |     | 250 |     |     |     | 255 |
| 5  | Leu | Ile | Gly | Pro | Pro | Leu | Leu | Ile | Pro | Met | Tyr | Phe | Gln | Tyr |
|    |     |     |     | 260 |     |     |     |     |     | 265 |     |     |     | 270 |
|    | Ile | Ile | Met | Thr | Met | Ile | Val | His | Lys | Asn | Trp | Val | Asp | Leu |
|    |     |     |     | 275 |     |     |     |     |     | 280 |     |     |     | 285 |
|    | Trp | Ala | Val | Ser | Tyr | Tyr | Ile | Arg | Phe | Phe | Ile | Thr | Tyr | Ile |
|    |     |     |     | 290 |     |     |     |     |     | 295 |     |     |     | 300 |
| 10 | Phe | Tyr | Gly | Ile | Leu | Gly | Ala | Leu | Leu | Phe | Leu | Asn | Phe | Ile |
|    |     |     |     | 305 |     |     |     |     |     | 310 |     |     |     | 315 |
|    | Phe | Leu | Glu | Ser | His | Trp | Phe | Val | Trp | Val | Thr | Gln | Met | Asn |
|    |     |     |     | 320 |     |     |     |     |     | 325 |     |     |     | 330 |
| 15 | Ile | Val | Met | Glu | Ile | Asp | Gln | Glu | Ala | Tyr | Arg | Asp | Trp | Phe |
|    |     |     |     | 335 |     |     |     |     |     | 340 |     |     |     | 345 |
|    | Ser | Gln | Leu | Thr | Ala | Thr | Cys | Asn | Val | Glu | Gln | Ser | Phe | Phe |
|    |     |     |     | 350 |     |     |     |     |     | 355 |     |     |     | 360 |
|    | Asp | Trp | Phe | Ser | Gly | His | Leu | Asn | Phe | Gln | Ile | Glu | His | His |
|    |     |     |     | 365 |     |     |     |     |     | 370 |     |     |     | 375 |
| 20 | Phe | Pro | Thr | Met | Pro | Arg | His | Asn | Leu | His | Lys | Ile | Ala | Pro |
|    |     |     |     | 380 |     |     |     |     |     | 385 |     |     |     | 390 |
|    | Val | Lys | Ser | Leu | Cys | Ala | Lys | His | Gly | Ile | Glu | Tyr | Gln | Glu |
|    |     |     |     | 400 |     |     |     |     |     | 405 |     |     |     | 410 |
| 25 | Pro | Leu | Leu | Arg | Ala | Leu | Leu | Asp | Ile | Ile | Arg | Ser | Leu | Lys |
|    |     |     |     | 415 |     |     |     |     |     | 420 |     |     |     | 425 |
|    | Ser | Gly | Lys | Leu | Trp | Leu | Asp | Ala | Tyr | Leu | His | Lys | Xaa | Ser |
|    |     |     |     | 430 |     |     |     |     |     | 435 |     |     |     | 440 |
|    | Ser | Pro | Arg | Asp | Thr | Val | Gly | Lys | Gly | Cys | Arg | Trp | Gly | Asp |
|    |     |     |     | 445 |     |     |     |     |     | 450 |     |     |     | 455 |
| 30 | Gln | Arg | Asn | Asp | Gly | Leu | Leu | Phe | Xaa | Gly | Val | Ser | Glu | Arg |
|    |     |     |     | 460 |     |     |     |     |     | 465 |     |     |     | 470 |
|    | Val | Tyr | Ala | Leu | Leu | Thr | Asp | Pro | Met | Leu | Asp | Leu | Ser | Pro |
|    |     |     |     | 475 |     |     |     |     |     | 480 |     |     |     | 485 |
| 35 | Leu | Leu | Ser | Phe | Phe | Ser | Ser | His | Leu | Pro | His | Ser | Thr | Leu |
|    |     |     |     | 490 |     |     |     |     |     | 495 |     |     |     | 500 |
|    | Ser | Trp | Asp | Leu | Pro | Ser | Leu | Ser | Arg | Gln | Pro | Ser | Ala | Met |
|    |     |     |     | 505 |     |     |     |     |     | 510 |     |     |     | 515 |
|    | Leu | Pro | Val | Pro | Pro | Ser | Pro | Phe | Phe | Gln | Gly | Ala | Glu | Arg |
|    |     |     |     | 520 |     |     |     |     |     | 525 |     |     |     | 530 |
| 40 | Pro | Pro | Gly | Val | Ala | Leu | Ser | Tyr | Leu | His | Ser | Leu | Pro | Leu |
|    |     |     |     | 535 |     |     |     |     |     | 540 |     |     |     | 545 |
|    | Met | Gly | Gly | Asp | Gln | Arg | Ser | Met | Gly | Leu | Ala | Cys | Glu | Ser |
|    |     |     |     | 550 |     |     |     |     |     | 555 |     |     |     | 560 |
| 45 | Leu | Ala | Ala | Trp | Ser | Leu | Gly | Ile | Thr | Pro | Ala | Leu | Val | Leu |
|    |     |     |     | 565 |     |     |     |     |     | 570 |     |     |     | 575 |
|    | Met | Leu | Leu | Gly | Phe | Ile | Gly | Ala | Gly | Pro | Ser | Arg | Ala | Gly |
|    |     |     |     | 580 |     |     |     |     |     | 585 |     |     |     | 590 |
|    | Leu | Thr | Leu | Pro | Ala | Trp | Leu | His | Ser | Pro | Xaa | Arg | Leu | Pro |
|    |     |     |     | 595 |     |     |     |     |     | 600 |     |     |     | 605 |
| 50 | Val | His | Pro | Phe | Ile | Glu | Arg | Pro | Ala | Leu | Leu | Gln | Ser | Ser |
|    |     |     |     | 610 |     |     |     |     |     | 615 |     |     |     | 620 |
|    | Leu | Pro | Pro | Ala | Ala | Arg | Leu | Ser | Thr | Arg | Gly | Leu | Ser | Xaa |
|    |     |     |     | 625 |     |     |     |     |     | 630 |     |     |     | 635 |
|    | Val | Gln | Gly | Pro | Arg | Pro | Ala | Gly | Thr | Ala | Ser | Pro | Asn | Leu |
|    |     |     |     | 640 |     |     |     |     |     | 645 |     |     |     | 650 |
| 55 | Pro | Trp | Lys | Ser | Pro | Pro | Pro | His | His | Xaa | Ser | Ala | Leu | Thr |
|    |     |     |     | 655 |     |     |     |     |     | 660 |     |     |     | 665 |
|    | Gly | Phe | His | Gly | Pro | His | Ser | Thr | Ala | Ser | Pro | Thr | Xaa | Ala |
|    |     |     |     | 670 |     |     |     |     |     | 675 |     |     |     | 680 |
| 60 | Asp | Leu | Gly | Thr | Lys | Gly | Gly | Val | Pro | Arg | Leu | Leu | Xaa | Leu |
|    |     |     |     | 685 |     |     |     |     |     | 690 |     |     |     | 695 |
|    | Arg | Gly | Ser | Gly | His | Val | Gln | Gly | Gly | Ala | Gly | Trp | Pro | Gly |
|    |     |     |     | 700 |     |     |     |     |     | 705 |     |     |     | 710 |
| 65 | Ser | Ala | His | Pro | Pro | Ala | Phe | Pro | Gln | Gly | Val | Leu | Arg | Ser |
|    |     |     |     | 715 |     |     |     |     |     | 720 |     |     |     | 725 |
|    | Ile | Leu | Glu | Gln | Ser | Asp | Pro | Ser | Pro | Lys | Ala | Leu | Leu | Ser |
|    |     |     |     | 730 |     |     |     |     |     | 735 |     |     |     | 740 |
|    | Gly | Gln | Cys | Gln | Pro | Ile | Pro | Gly | His | Leu | Ala | Pro | Gly | Asp |
|    |     |     |     | 745 |     |     |     |     |     | 750 |     |     |     | 755 |
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tgtaaaacga cggccagt 18

<210> 38  
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gaaacagcta tgaccatg

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/22692

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/53 C12N9/02 C12N15/86 C12P7/64 C12N5/10  
 C11B1/00 A61K31/20 A61K31/23 A61K7/00 A23K1/00  
 A23L1/30 //(C12P7/64, C12R1:91)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P A61K C11B A23K A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| P, X       | WO 98 46765 A (THURMOND JENNIFER ; CALGENE LLC (US); ABBOTT LAB (US); KNUTZON DEBO)<br>22 October 1998 (1998-10-22)<br>abstract<br>see examples<br>page 30 | 1-75                  |
| X          | WO 96 21037 A (MARTEK BIOSCIENCES CORP ; KYLE DAVID J (US))<br>11 July 1996 (1996-07-11)   | 74, 75                |
| A          | see claims<br>abstract   | 2                     |
|            | -/-  |                       |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

16 February 2000

Date of mailing of the international search report

22/02/2000

Name and mailing address of the ISA

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 Fax (+31-70) 340-3016

Authorized officer

Lejeune, R

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/22692

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| X          | MICHAELSON L ET AL: "Isolation of a delta5-fatty acid desaturase gene from <i>Mortierella alpina</i> "<br>JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 273, no. 30, 24 July 1998 (1998-07-24), pages 19055-19059, XP002076636<br>ISSN: 0021-9258   | 68,69,74              |
| A          | abstract<br>page 19059, column 2, paragraph 3   | 1,2                   |
| P,X        | KNUTZON D S ET AL: "Identification of Delta5 - desaturase from <i>Mortierella alpina</i> by heterologous expression in Bakers yeast and canola"<br>JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 273, no. 45, 6 November 1998 (1998-11-06), pages 29360-29366, XP002106760<br>ISSN: 0021-9258 | 68,69,74              |
| A          | abstract  | 1,2                   |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 22692

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 3,22,23,30,49,50  
are directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark n Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 99/22692

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| WO 9846765 A                              | 22-10-1998          | US 5972664 A               | 26-10-1999          |
|   |                     | AU 7114798 A               | 11-11-1998          |
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|   |                     | WO 9846764 A               | 22-10-1998          |
| WO 9621037 A                              | 11-07-1996          | US 5658767 A               | 19-08-1997          |
|   |                     | AU 713567 B                | 02-12-1999          |
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|   |                     | BR 9607179 A               | 11-11-1997          |
|   |                     | CA 2209513 A               | 11-07-1996          |
|   |                     | CN 1175976 A               | 11-03-1998          |
|   |                     | DE 800584 T                | 06-05-1999          |
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|   |                     | NO 973085 A                | 03-09-1997          |
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